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Biochemical Changes in Mouse Lung Concomitant with Influenza Virus Infection.* (21051)

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(Introduced by A. F. Rasmussen, Jr.)

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In an attempt to gain information concerning the metabolic interrelationships between the host cells of mouse lung and the invasion and proliferation of influenza virus, studies of the *in vitro* metabolism of both normal and of Type A, PR8 influenza virus infected mouse lung were carried out. The enzymic activities of normal and virus infected lung tissue homogenates were compared by measuring the oxygen uptakes consequent upon the addition of various substrates. A stimulated or characteristically altered metabolic rate in infected tissue might reflect a diversion of normal host metabolism toward viral synthesis. Accordingly, compounds generally associated with providing energy for the synthesis of protoplasm and compounds assumed to be important in nucleic acid metabolism were investigated. Since it was found that the activity of xanthine oxidase was increased 2 to 2.5 times in the infected tissue, experiments were carried out to determine if this activity was due to an increase in the level of the same kind of enzyme in the tissue, or, if the presence of the proliferating virus brought about a difference in kind of enzyme. The effect produced by several enzyme poisons on the activity of xanthine oxidase in normal lung was compared with that of the poison-effect on the enzyme activity in infected lung. The influence of methylene blue on the 2 systems was investigated, presumably, to detect possible differences between the "dehydrogenase" and "oxidase" parts of the enzyme molecule(1). Assays for possible end-products of hypoxanthine-xanthine oxidations were made and the results compared.

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These experiments were essentially exploratory in nature, intended to initiate a series of experiments designed to elucidate the role of host tissue metabolism in the process of influenza virus proliferation.

Methods and materials. The Type A, PR8 strain of influenza virus used throughout these investigations was obtained originally from Dr. Carl J. DeBoer at Birmingham Veterans Hospital, Van Nuys, Calif. During the course of this work, the virus has been through 63 mouse lung passages and was sufficiently pathogenic for 4- to 6-week-old albino Swiss mice weighing from 10 to 16 g so that the intranasal inoculation of up to a 10^{-4} dilution led to a fatal disease associated with extensive pneumonia. The animals were infected intranasally under light ether anesthesia, with an inoculum of 0.03 ml of a 10^{-3} dilution of infected lung tissue (10 to 100 lethal doses). Lung tissue homogenate from paired normal and 48- to 72-hour infected mice was used as a source of enzyme. The mice were killed by a sharp blow to the head, the lungs excised and placed on a moistened piece of sterile filter paper contained in a petri-dish packed in crushed ice. The tissue was trimmed, blotted free of excess blood and ground in 4 ml ice-cold phosphate buffer at pH 7.4 in the type of homogenizer developed by Brendler(2). The lungs of 6 mice were pooled for each experiment excepting where differences between individual animals were under investigation. The presence of virus was established by animal inoculation and by the hemagglutination method of Hirst as modified by Salk(3). The enzymic activity of the homogenate was determined manometrically by means of a conventional Warburg apparatus, xanthine oxidase according to the method of Axelrod and Elvehjem(4) and the effect of methylene blue on the rate of oxygen consumption in the text mixtures according to Rickert and Westerfeld(1). The difference between the respiration of the homogenate in the presence and absence of substrate was calculated and recorded as activity produced by a given substrate. All Warburg flasks used in the determinations contained 1.5 ml of homogenate (equivalent on an average, to 15 mg dry weight) in a total fluid volume of 3.2 ml at a pH of 7.4 and containing 250 μg of streptomycin plus 1500 units of penicillin. After many of the Warburg experiments assays for certain end-products were made on the flask contents; uric acid determinations were made according to the method of Folin as modified by Heplar and Stoskope(5); urea determinations by the method of Karr as described by Hawk *et al.*(6); and ammonia determinations by the method of Van Slyke and Cullen described by Hawk *et al.*(6). The inhibition of xanthine oxidase by tetraethylthiuram disulphide (Antabuse) was investigated by the method of Rickert *et al.*(7) and that of 2-amino-4-hydroxy-6-pteridinecarbonzaldehyde (6-pteridylaldehyde) according to Kalcar as described by Van Meter and Oleson(8).

Results. Results of experiments with compounds, the oxidative decomposition of which may give rise to energy yielding mechanisms, are presented in Table I. The substrates were used as their sodium salts where feasible, 10 μM in 0.2 ml volume being used routinely. The values recorded are the average of at least 3 experiments and represent the oxygen uptake of 20 mg tissue (dry weight equivalent) per 20 minutes stimulated by the addition of substrate. In each experiment the

TABLE I. Oxygen Consumption of Normal and of PR8 Influenza Virus Infected Mouse Lung upon Addition of Compounds Capable of Yielding Energy.

Compound tested	μl O_2 uptake/20 min./20 mg dry lung Normal lung	Infected lung
Formate	1	1
Acetate	2	2
Lactate	2	6
Citrate	0	0
Cis-aconate	1	2
Alpha-ketoglutarate	4	4
Succinate	16	12
Fumarate	4	2
Maleate	0	0
Malate	0	3
Malonate	3	3
Pyruvate	2	2
Oxalacetate	1	0
Ribose	0	0
Glucose	1	1
Fructose-di-phosphate	0	0
Alpha-glycerophosphate	1	3
Glycerol	1	2
Benzoate	1	3
Glutamate	0	0
Aspartate	0	0
l-Histidine	0	0
l-Proline	0	0
l-Alanine	0	0
dl-Serine	2	0
Glycine	0	0

oxygen uptake representing the endogenous respiration was subtracted from the reported values. In spite of the fact that there are some differences between the values obtained for the 2 different homogenates, the total over-all values are of such low order as to make the significance of these figures doubtful. Of particular interest was the finding that, on a comparative basis, succinate produced the greatest stimulation in oxygen uptake in both normal and infected tissue and that this stimulation was greater in normal tissue. Ackermann(9) has found that the multiplication of influenza virus is inhibited in chick embryo chorioallantoic tissue cultures by malonic acid and by Antimycin A; both succinic dehydrogenase inhibitors. If succinate oxidation contributes to the over-all process of influenza virus multiplication, then this enzymic reaction is readily available in mouse lung.

The results of experiments with compounds related to nucleic acid metabolism are presented in Table II. The purine and pyrimidine bases as well as the nucleosides were employed at a final concentration of 10 μM per flask, the nucleotides at 5 μM and the thymus nucleic acid at 2.5 μM per flask. It is apparent that any demonstrable activity in normal lung was increased 2 to 2.5 times in the virus infected tissue. In some cases actual decrease in oxygen consumption was observed, *i.e.*, the oxygen uptake was less in the flasks with substrate than in the endogenous flasks. The addition of adenine generally inhibited oxygen uptake as did the addition of orotic acid. The inhibition caused by adenine provides for interesting speculation when one considers that adenine is a part of the xanthine oxidase molecule. It is believed that the oxygen uptake was, in a large measure, due to xanthine oxidase activity since the other steps, so far as is known in the breakdown of these compounds, are hydrolytic rather than oxidative(10). Most likely the compounds were degraded to hypoxanthine or xanthine and thence oxidized to uric acid. In order to test this hypothesis, assays for uric acid(5) were made on the flask contents of

TABLE II. Enzymic Activity of Purines and Pyrimidines and Other Compounds Related to Nucleic Acid Metabolism in Normal and in PR8 Influenza Infected Mouse Lung.

Compound tested	μl O_2/20 min./20 mg dry lung* Normal lung	Infected lung
Hypoxanthine	6	13
Inosine	6	12
Xanthine	4	10
Xanthosine	3	7
Adenine†	0	0
Adenosine	5	12
Adenylic acid	2	6
Guanine	0	3
Guanosine	1	4
Guanylic acid	0	2
Uric acid	0	0
Cytosine	0	0
Cytidine	0	0
Cytidylic acid	0	0
Uracil	0	0
Uridylic acid	0	0
Thymine	0	0
Orotic acid†	0	0
Thymus nucleic acid	0	0

* Recorded values are average of at least 3 experiments and calculated after endogenous oxygen uptake had been subtracted.

† O_2 uptake was actually lower than endogenous upon addition of these substrates.

TABLE III. Influence of Methylene Blue (M.B.) on Oxygen Uptake Stimulated by Purine Compounds Showing Enzymic Activity and Resultant Accumulation of Uric Acid.

	Normal lung				Infected lung			
	O_2 uptake*		Uric acid†		O_2 uptake		Uric acid	
Compound tested	—	+M.B.‡	—	+M.B.	—	+M.B.	—	+M.B.
Hypoxanthine	7	14	0	.03	13	21	.03	.24
Inosine	6	12	0	.14	11	19	.18	.30
Xanthine	5	9	.03	.36	10	16	.36	.45
Xanthosine	4	6	.02	.09	7	11	.10	.34
Adenosine	5	9	0	.11	12	17	.12	.20
Adenylic acid	2	4	0	.03	6	9	.06	.14
Guanine	1	3	0	.07	4	6	.03	.09

* μl/20 min./20 mg dry lung.

† Mg accumulating/ml of flask content at end of 100 min. incubation.

‡ Methylene blue, 0.2 ml of 0.0113 M solution was added as a part of the 3.2 ml flask fluid volume.

the flasks showing an increase in oxygen consumption. A protein-free filtrate was prepared and treated with a specific uric acid reagent in the presence of optimal amounts of urea and sodium cyanide. The blue color produced by the reduction of alkaline phosphotungstate by urate was determined in a photoelectric colorimeter. In addition, since it has been demonstrated that the addition of methylene blue to such a test-system increases the activity of xanthine oxidase(1), an investigation was made of the influence of the dye on oxygen consumption stimulated by addition of these substrates and additional assays for uric acid made on the flask contents. The results of these experiments are recorded in Table III. In the flasks showing maximal oxygen uptake there was also an increase in the amount of uric acid formed. Also, where there was enzymic activity, the addition of methylene blue increased this activity. Assays for ammonia(6) carried out by treating the flask contents with an equal volume of saturated carbonate solution and transferring the liberated ammonia by distillation into a boric acid receiving solution where it was titrated directly with a standard acid solution, revealed that in the flasks showing maximal oxygen uptake there was a small increase in the amount of ammonia produced; from 0.2 to 3.3 mM per ml of flask content. Uricase was not found in mouse lung.

Results of the experiments recorded in Table IV provide additional evidence that the activity of xanthine oxidase is increased in infected lung. It can be seen that methylene blue apparently increased the enzymic activity in both normal and infected tissue; the addition of dye did not elicit a differential effect. Table V indicates the effect of cyanide on the xanthine oxidase found in virus infected lung and the results of attempts to reverse this effect with either cytochrome *c* or ferrous sulphate. In the flasks without methylene blue, the inhibition was irreversible, *i.e.*, by either cytochrome *c* or ferrous ions. In flasks containing methylene blue the inhibition was partially reversed by either cytochrome *c* or ferrous sulphate. This does not necessarily indicate that the xanthine oxidase is linked to

TABLE IV. Influence of Methylene Blue (M.B.) on Xanthine Oxidase Activity in 6 Normal and 6 Influenza Virus Infected Mouse Lung Homogenates.

	μl oxygen consumed/20 min./20 mg dry lung					
	Xanthine		Hypoxanthine		O_2 increase in M.B.	
	−M.B.	+M.B.	−M.B.	+M.B.	Xant.	Hypo-xant.
	Infected lungs					
	5.5	10.8	8.8	22.6	5.3	13.8
	5.8	21.2	10.0	20.2	15.4	10.2
	11.1	29.0	12.6	28.1	17.9	15.5
	12.2	30.4	14.4	33.4	18.2	29.0
	14.1	27.1	14.6	27.9	13.0	13.3
	15.5	29.3	19.7	37.0	13.8	17.3
Avg	10.7	24.6	13.3	28.2	14.3	14.9
	Normal lungs					
	2.3	10.4	2.8	9.3	8.1	6.5
	3.6	16.2	4.0	10.4	12.6	6.4
	3.9	24.6	3.8	19.6	20.7	15.8
	4.2	9.5	5.3	13.9	5.3	8.6
	5.1	16.8	5.0	15.0	11.7	10.0
	5.9	11.1	6.5	11.6	5.2	5.1
Avg	4.2	14.8	4.6	13.3	10.6	8.7

TABLE V. Effect of HCN on Xanthine Oxidase Activity in Mouse Lung Infected with PR8 Influenza Virus and Reversal of This Effect by Cytochrome *C* and by Ferrous Ions.

Compounds tested*	μl O_2 uptake/20 min./20 mg dry lung −M.B.	% inhibition	+M.B.	% inhibition
Controls	10.6		22.6	
10^{-3} M HCN*	1.3	88	3.0	87
Idem + 10^{-3} M cytochrome *c*†	1.6	85	9.0	61
Idem + 10^{-3} M $FeSO_4$†	1.3	88	8.0	65

* The correct mixtures of KOH-KCN were employed in the center-wells to give this concentration of HCN in the flasks.

† Concentrations are number of moles/ml of final fluid volume.

the cytochrome system since the same result can be obtained by substituting ferrous sulphate for the cytochrome, however, it has been shown that cytochrome *c* can be reduced by xanthine oxidase(11,12). The results of experiments with other inhibitors, *i.e.*, azide, arsenite, monoiodoacetate and monofluoroacetate are tabulated in Table VI. Azide, in the lowest dilution used, inhibited the oxygen uptake by 40%; in the presence of methylene blue this inhibition was reduced in 12%. The highest dilution of azide produced a similar, although greatly diminished, effect. Likewise, in the absence of methylene blue, arsenite at 0.0005 M concentration produced 89 and 73% inhibition respectively; 0.0005 M concentration produced 89 to 61% inhibition. Iodoacetate in 0.01 M concentration reduced the activity of the xanthine oxidase by 100% when methylene blue was not present; addition of dye reduced this value by one-half. Fluoroacetate, by comparison, was not a potent inhibitor of this system either in the presence or absence of methylene blue, in fact, it produced a 13% stimulation in oxygen uptake in the homogenate to which the dye had been added. Antabuse did not prove to be a potent inhibitor in this system. On the other hand, 6-pteridyl aldehyde at optimal concentrations completely inhibited the enzyme and the addition of methylene blue did not alter the result, thus indicating action at the substrate level and the prevention of hypoxanthine and xanthine activation. Since the results with these inhibitors on the enzyme found in infected tissue were similar to the results with normal tissue, details of the experiments with the latter will not be reported here.

Assays were made for uric acid, ammonia, and urea accumulating either directly or indirectly from xanthine oxidase activity. When hypoxanthine was added as substrate the amount of uric acid accumulating per Warburg flask after 100 minutes incubation ranged from zero to 0.30 mg per flask. If xanthine were the added substrate then the resulting uric acid values ranged from 0.03 to 0.54 mg per flask. The addition of methylene blue substantially increased the amount of uric acid formed; this effect being more marked when hypoxanthine was the added substrate. The accumulation of uric acid revealed no qualitative difference in the xanthine oxidase activity; the enzyme from both normal and infected lung oxidized the hypoxanthine or xanthine to uric acid in proportion to the amount of enzyme present in the tissue. The results of ammonia assays on both normal and infected tissue failed to show an accumulation of the product resulting from the enzymic degradation of hypoxanthine or xanthine. Assays for urea production(6), made by converting the urea in protein-free filtrates to ammonium car-

TABLE VI. Effects of Sodium Azide, Sodium Arsenite, Sodium Monoiodoacetate and Sodium Monofluoroacetate on Xanthine Oxidase Activity of Mouse Lung Infected with PR8 Influenza Virus.

Compounds tested*	μl O_2 uptake/20 min./20 mg dry lung −M.B.	% inhibition	+M.B.	% inhibition
Controls	14.2		33.0	
Azide M/ 2000	8.5	40	28.9	13
" M/10000	11.3	21	30.5	8
Arsenite M/ 200	.0	100	9.2	73
" M/2000	1.6	89	12.8	61
Controls	10.9		26.6	
Iodoacetate M/ 100	.0	100	13.3	50
" M/1000	9.2	16	24.6	8
Fluoroacetate M/100	9.3	16	23.4	10
" M/10000	11.6	0	30.1	0

* Inhibitor and homogenate were incubated for 20 min. before the substrate was tipped-in. Concentrations are number of moles/ml of final fluid volume.

TABLE VII. Correlation of Change in Virus Concentration with Change in Levels of Xanthine Oxidase Activity in Mouse Lung after Infection with PR8 Influenza Virus.

	*Group 1			Group 2			Group 3			Average		
Time†	E.A.‡	H.T.§	U.A.‖	E.A.	H.T.	U.A.	E.A.	H.T.	U.A.	E.A.	H.T.	U.A.
0	1.0	0	.0	1.	0	.0	2.0	0	.0	1.3	0	.0
24	2.3	80	.06	8.	20	.18	2.0	20	.09	4.1	40	.11
48	3.0	80	.12	7.	640	.18	6.6	640	.18	5.5	453	.16
72	9.3	1280	.24	7.3	1280	.30	10.6	1280	.27	9.1	1280	.27
96	10.0	640	.30	7.3	40	.21	1.0	0	.09	4.8	226	.20

* Group of 5 mice, one chosen at random for examination at the indicated time.

† No. of hours between initial infection and titration.

‡ Enzyme activity: μl O_2/20 min./20 mg dry lung. (Xanthine was added as substrate.)

§ Hirst titer: the highest dilution of virus lung showing complete hemagglutination.

‖ Uric acid: total amount of compound per flask at end of experiment (100 min.) in mg.

bonate by the action of added urease, Nesslerizing in the presence of gum gatti and readings in a photoelectric colorimeter, did not show urea to be an end-product of the xanthine oxidase activity. Also, assays made on the unincubated homogenates failed to detect any difference between the *in vivo* urea content of normal and infected tissue.

An attempt was made to correlate the more quantitative aspects of virus multiplication with the increase in enzyme level; for purposes of convenience these were called "progression experiments." Results recorded in Table VII show a correlation between the day-by-day multiplication of virus in the mouse lung, as determined by the hemagglutination titer, and the change in enzyme level in the same lung, as determined by oxygen uptake and by uric acid production.

It was of interest to investigate the level of liver xanthine oxidase in mice concomitant with influenza virus infection. Therefore, liver and lung from the same animals were processed and examined in the same manner. While the lung enzyme from the infected mice showed a 2.5 fold interest in activity over that of the non-infected mice, the liver xanthine oxidase remained at the same level of activity as that of the non-infected animals.

Comment. The addition of any one of 26 compounds, the oxidation of which might possibly yield energy for the formation of influenza virus particles to normal and to virus infected mouse lung tissue homogenate revealed only minor enzymic differences between the 2 homogenates. Apparently, the propagation of influenza virus in mouse lung does not alter to a significant degree the level of host tissue enzymes concerned in the oxidation of these compounds. These experiments are in no sense complete since no attempt was made to follow in detail the reactions which were measured only by the rate of oxygen uptake. The question of energensis in the formation of virus particles is an elusive one. Certain information indicates that a functioning Krebs cycle is essential for the propagation of influenza virus both in mouse lung and chorioallantoic membrane tissue cultures(9,13). However, whether the parasite requires energy over and above that required for the normal cellular metabolic reactions of the host tissue remains to be established(14).

Since virus particles are composed of complex nucleoprotein material, the synthesis of which is stimulated by the entrance of virus particles into the host cell, it seems likely that at least a part of the increased *in vitro* activity in the virus infected lung caused by the addition of certain purine bases, nucleosides and nucleotides is indicative of special enzyme activity brought about by the presence of the viral infection. The greatest part of the oxygen uptake resulting from the metabolism of these compounds is probably due to xanthine oxidase activity as shown by the resultant accumulation of uric acid and ammonia and by the effect of methylene blue on the system. If this is true, then it serves as indirect evidence that certain purine deaminases, nucleosidases and nucleotidases are increased in the influenza virus infected mouse lung. Also, the importance of xanthine oxidase in the purine metabolism of mouse lung seems evident.

Although the results of the experiments recorded here clearly show that this infectious process is accompanied by an increase in the level of xanthine oxidase, the exact role of the enzyme remains to be determined. The "progression experiments" suggest that the enzymic activity is more than a reflection of an inflammatory process excited by the presence of virus in the tissue. In mice receiving 10 to 100 lethal doses of infecting virus, the increment and decrement periods in the level of xanthine oxidase activity roughly parallel the virus increment and decrement periods. Of possible significance here is the finding by Ackerman(15) that methionine is involved in the biosynthesis of influenza virus; the same amino acid is required in a diet favorable for the biosynthesis of xanthine oxidase(16,17). Bauer(18) reported a correlation between yellow fever infection and the development of xanthine oxidase in chick embryos. On the other hand, the role of hypoxanthine and xanthine in the purine metabolism of animals has not been considered important(19); however, more recent experiments tend to show that hypoxanthine may occupy a place of central importance in the metabolism of purine containing components in nucleic acids (20).

In an attempt to resolve the questions raised by the results of the experiments reported here, further experiments are contemplated to determine: (a) if non-specific inflammatory processes in mouse lung are accompanied by an elevation in the level of xanthine oxidase; (b) if the increased enzyme level indicates an increased breakdown of host nucleic acids, the end-products of which might be hypoxanthine and xanthine; and (c) if the action of xanthine oxidase is essential to the biosynthesis of influenza virus.

Summary. 1. Mouse lung tissue possesses the capacity to oxidize several of the naturally occurring compounds which are capable of undergoing oxidative decomposition and giving rise to energy-yielding mechanisms. Under the limiting conditions of these exploratory experiments, the presence of a Type A, PR8 influenza virus infection in this tissue does not appear to alter, to a significant degree, these oxidative reactions. 2. Influenza infection appears to increase the capacity of host lung tissue to metabolize several compounds concerned in nucleic acid metabolism. Whether this phenomenon is indicative of cellular breakdown incidental to the viral infectious process or whether it represents an "active" degradation of specific molecules consequent to virus synthesis is still to be determined. 3. Xanthine oxidase appears to play a central role in the *in vitro* purine metabolism of mouse lung; the activity of this enzyme is increased in the infected tissue. The increment and decrement periods of virus proliferation roughtly parallel the increment and decrement periods of enzyme activity. In no case was a qualitative difference found between the xanthine oxidases of normal and virus infected lung tissue; the effects produced by the addition of metabolic inhibitors, the influence of methylene blue, and the end-products of the reactions were the same. The significance of these findings has been discussed.

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A Non-Auxinic Growth-Promoting Factor Present in Crown Gall Tumor Tissue. (21052)

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An abundance of evidence published during the past decade has suggested that crown gall tumor cells proliferate abnormally because they elaborate in greater than regulatory amounts a growth-promoting substance(1-3). Although auxin synthesized by the tumor cell has commonly been regarded as playing an etiological role in abnormal growth of such cells, it is by no means certain that the characteristic behavior of the crown gall tumor cell can be explained solely or even in large part on the basis of high and unregulated auxin levels in those cells. An attempt was therefore made to determine whether, in addition to growth substances of the auxin type, non-auxinic factors capable of stimulating cell proliferation are present in plant tumor tissues.

Methods and materials. In order to investigate this problem it appeared desirable to use as a test object plant cell types that would not proliferate actively in White's culture medium containing auxin. Steward and Caplin(4) have recently reported that mature parenchymatous cells of the potato (*Solanum tuberosum* L.) are not stimulated to active cell division by 2,4-dichlorophenoxyacetic acid. These workers found, however, a synergistic effect to exist between 2,4-D and coconut milk in promoting cell division in the potato parenchyma tissue. Tobacco (*Nicotiana tabacum* L.) pith cells have been reported(5) to enlarge but not to divide under the influence of indole acetic acid. Tobacco pith tissue, which was used in this study, was isolated aseptically from the middle third of greenhouse-grown tobacco plants that were about 3 feet tall. Pith tissue fragments free of internal phloem were cultured either on White's basic medium containing 1% agar(6) or on that medium supplemented with the desired concentration of an auxin and/or tumor extract. The tumor extracts were obtained from sterile crown gall tissue grown in culture. The tumors were harvested during the period of active growth and placed in a Waring Blendor at 4°C. They were thoroughly macerated, pressed through cheesecloth, and centrifuged for 10 minutes at 7,000 r.p.m. The supernatant fluid was incorporated in White's medium in a concentration of 15% by volume in certain experiments described below. The pith tissues were cultured in 50 ml Erlenmeyer flasks containing a total of 14 ml of media. The cultures were maintained at room temperature in diffuse light.

Results. Tobacco pith tissue planted on White's basic medium showed a very limited or, in most instances, no wound healing response even after prolonged incubation, Fig. 1A. In the presence of suitable concentrations of growth substances of the auxin type, however, the volume of the pith tissue more than doubled in a 10-day period, Fig. 1B. The tissue frequently became contorted as if under considerable internal stress. Subsequently, the structure of the tissue itself showed a striking tendency to disintegrate. Enlarged pith cells projected in all directions from what was originally a compact, well organized tissue fragment. In accord with findings reported earlier(5) it was found that proliferation of the pith cells did not occur in the presence of the auxins used. In these experiments naphthalene acetic acid, indole butyric acid, indole acetic acid, and para-

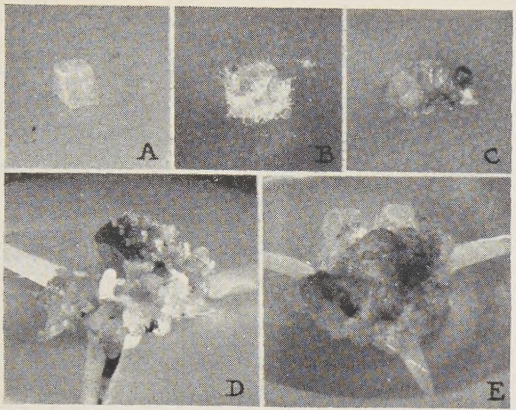

FIG. 1. Tobacco pith tissue planted on: A, White's basic medium; B, White's basic medium containing naphthalene acetic acid in a concentration equal to 1.0 mg/liter; C, White's medium containing 15% *Vinca* tumor tissue extract and naphthalene acetic acid in a concentration of 0.01 mg/liter. The photograph was taken 4 weeks after pith tissue was planted on the medium. D, same tissue shown in C after an additional 4 weeks of growth. Note the organization of leaves of a primitive type arising from the tissue mass. E, White's medium containing 15% *Vinca* tumor extract and naphthalene acetic acid in a concentration of 0.1 mg/liter. The photograph was taken 4 weeks after the pith tissue was planted on the medium. Compare with A and C.

chlorophenoxyacetic acid were tested separately in White's medium in concentrations equal to 100, 10, 1.0, 0.1, 0.01 mg/liter. Naphthalene acetic acid and indole butyric acid were found to be most effective in inducing cell enlargement with resulting contortion of the pith tissue when used in concentrations equal to 10 mg and 1.0 mg/liter. At a concentration equal to 0.1 mg/liter the activity of the 2 growth substances was somewhat less pronounced but still considerable. Indole acetic acid and parachlorophenoxyacetic acid, on the other hand, were found to be far less effective at all concentrations tested.

Since tobacco pith cells did not respond with active cell division to growth substances of the auxin type, an attempt was made to determine whether extracts of crown gall tumor tissue when incorporated in White's basic medium contained a factor or factors that would encourage the active proliferation of tobacco pith cells.

Although in preliminary experiments extracts of crown gall tumors originally isolated from such taxonomically widely separated plant species as *Vinca rosea,* Parthenocissus, and cactus were tested and found to possess varying degrees of biological activity, the studies described below were carried out with the use of extracts obtained from *Vinca rosea* tumors. The *Vinca* tumor extract, which was found to be highly effective, was in all instances incorporated in White's basic medium at a concentration of 15% by volume. Unless otherwise stated the medium was sterilized in an autoclave at 10 lb pressure for 12 minutes. Where used, auxin in the form of naphthalene acetic acid was added aseptically to the medium in the desired concentration following sterilization.

The results of the experiments, which are in part pictured in Fig. 1, demonstrate that within limits a direct correlation exists between the concentration of naphthalene acetic acid in White's culture medium containing 15% tumor extract and the amount of proliferation of the tobacco pith cells. In the presence of a concentration of naphthalene acetic acid equal to 1.0 mg/liter in the tumor-extract-containing culture medium a very active proliferation of the pith cells resulted. At one-tenth this concentration of auxin, proliferation of the pith cells was less pronounced but still considerable (Fig. 1E). It is interesting to note that the growth pattern of the pith tissue in both instances showed, superficially at least, a striking resemblance to tobacco crown gall tumor tissue of the unorganized type grown on White's basic medium. When the concentration of auxin was reduced in the tumor-extract-containing medium to a level equal to 0.01 mg/liter, the tobacco pith tissue grew very slowly. Similar results were obtained in those instances in which auxin was not added to this medium. In further experiments, tumor extract sterilized by filtration with the use of a Selas porcelain filter, 0.03 porosity, and added aseptically to White's basic medium free of auxin failed to encourage proliferation of the tissue. The addition of auxin in a concentration of 0.1 mg or 1 mg/liter to this medium, however, permitted the rapid division of the pith cells. It is likely, therefore, that small amounts of auxin are formed as a result of the sterilization by autoclaving of the tumor extract. This trace of auxin together with the biologically active factor present in the tumor extract probably accounts for the limited proliferation of pith cells observed in the autoclaved medium to which no external source of auxin was applied.

It thus appears that neither the auxin nor the tumor extract factor can by itself cause normal tobacco pith to proliferate actively. When combined in the proper concentration, however, they are highly effective in accomplishing this end. These experiments have been repeated on 4 different occasions with similar results.

Of interest in these studies was the finding that tobacco pith tissue planted on White's medium containing tumor extract and very low concentrations of auxin grew slowly and in a more or less unorganized manner for a period of about 3-4 weeks, Fig. 1C. Thereafter, numerous small leaf-like structures developed from the tissue mass and in many instances covered the surfaces of the slowly proliferating tissues (Fig. 1D). Such tissues showed a resemblance to tobacco crown gall teratomata. Whether the pith tissue grows in an essentially unorganized manner or as a more or less organized teratoma-like growth appears to depend in large measure on the concentration of auxin present in the culture medium. It has thus been possible to reproduce under controlled conditions growth patterns which resemble superficially at least the 2 morphologically distinct types of crown gall tumors that have recently been described as occurring on tobacco(7). These artificially-stimulated pith tissues are, however, self-limiting and when the externally supplied stimuli are removed their growth commonly ceases entirely or, in some instances, progresses for a time at a very slow rate. Crown gall tumor tissue, on the other hand, is autonomous and is itself capable of synthesizing all of the growth factors necessary for its continued abnormal proliferation.

The biologically active factor found to be present in crown gall tumor tissue extract does not appear to be specifically associated with this plant tumor tissue. Its effect in stimulating the division of tobacco pith cells can be replaced in large part in an auxin-containing culture medium with 15% coconut milk or with an extract of young normal tips of tobacco plants. Since, however, this factor appears to be concerned specifically with cell division, it is likely that it plays a major role in the growth of crown gall tumor cells. Due cognizance should therefore be taken of the tumor extract factor in any consideration of the physiology of development of the crown gall tumor.

Summary. A biologically active factor is present in crown gall tumor tissue extracts which when used in association with an auxin is capable of encouraging the very active proliferation of normal tobacco pith tissue. Neither the factor present in the tumor extract nor the auxin is by itself effective in stimulating the active division of the pith cells.

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Inactivity of Dihydrouracil and β-Ureidopropionic Acid as Pyrimidine Precursors for Lactic Acid Bacteria. (21053)

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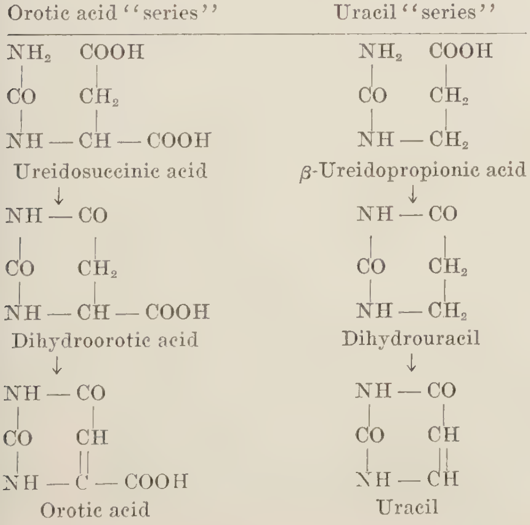
A recent study(1) showed that certain "pyrimidineless" lactobacilli may be classified into 3 groups (a) those (*Lactobacillus arabinosus* ATCC 8014 as an example) for which uracil rather than orotic acid is the "preferred" pyrimidine, (b) one (*Lactobacillus bulgaricus* 09) that utilizes orotic acid but not uracil, and (c) those (*Lactobacillus bulgaricus* ATCC 8001 as an example) for which uracil and orotic acid are equally active and essentially indistinguishable. These results at least suggest the possibility that there are 2 independent mechanisms for pyrimidine nucleotide or nucleic acid pyrimidine synthesis. One would appear to involve orotic acid while the other would appear to involve uracil. A number of intermediates in the biosynthesis of orotic acid have been established and include dihydroorotic acid(1,2) and ureidosuccinic acid(4-6). These compounds are active in supporting growth of *L. bulgaricus* 09 which utilizes orotic acid but not uracil. The corresponding compounds in a similar hypothetical biosynthesis of uracil are dihydrouracil and β-ureidopropionic acid.



This paper presents data to show that dihydrouracil and β-ureidopropionic acid are inactive in promoting growth of "pyrimidineless" lactobacilli that utilize uracil instead of orotic acid or utilize uracil and orotic acid equally well. The obvious conclusion from these studies is that orotic acid and uracil do not originate by "related" biosynthetic pathways.

Experimental. β-Ureidopropionic acid was synthesized by treatment of β-alanine methyl ester with KCNO according to the procedure of Lengfeld and Stieglitz(7). Dihydrouracil was synthesized from β-ureidopropionic acid by cyclization in acid solution(7). Both compounds as used were analytically correct. Dihydrouracil and β-ureidopropionic acid were examined for activity in promoting growth of *L. leichmannii* (ATCC 4797), *L. brevis* (ATCC 8287), *L. arabinosus* (ATCC 8014), *L. helveticus* (ATCC 335), *L. bulgaricus* 09, *L. bulgaricus* Sarles, *L. bulgaricus* (ATCC 8001), and *L. bifidus* (ATCC 4963) on media and under conditions described previously in detail(1). For *L. leichmannii, L. arabinosus,* and *L. helveticus* a pyrimidine source is only *stimulatory.* With these strains it was necessary to employ an incubation period of about 18 hours. For *L. brevis, L. bulgaricus* 09, *L. bulgaricus* Sarles, *L. bulgaricus* 8001, and *L. bifidus* a utilizable pyrimidine source is *essential.* With these strains an incubation period of 72-120 hours was used. The response to a pyrimidine source was determined by turbidimetric measurement with a Klett-Summerson photoelectric colorimeter.

Results. Both dihydrouracil and β-ureidopropionic acid are completely inactive in satisfying the pyrimidine requirements of the 8 strains of lactobacilli studied. Typical data demonstrating this point with *Lactobacillus bulgaricus* (ATCC 8001) are given in Table

TABLE I. Response of *Lactobacillus bulgaricus* (ATCC 8001) to Uracil, Dihydrouracil and β-Ureidopropionic Acid.

Compound (γ/10 ml)	Turbidity (Klett-Summerson units)
0	42
5 γ uracil	51
10	74
15	78
20	88
50	178
100	315
500	387
10 to 500 γ dihydrouracil	43
10 to 500 γ β-ureidopropionic acid	43

I. It is interesting to note that while this strain, for example, can utilize dihydroorotic acid presumably by unsaturation to orotic acid and can utilize uracil as well as orotic acid(1) it does not accomplish the unsaturation of dihydrouracil. In this connection Lieberman and Kornberg recently have shown(3) that dihydroorotic dehydrogenase from a bacterial source that is concerned with the reversible reaction: Orotic acid + DPNH + $H^+ \rightleftharpoons$ Dihydroorotic acid + DPN is inert towards uracil, cytosine, 5-methylcytosine, or thymine.

Summary. Dihydrouracil and β-ureidopropionic acid, the "uracil counterparts" of dihydroorotic acid and ureidosuccinic acid, intermediates in the biosynthesis of orotic acid, are inactive in promoting growth of 8 pyrimidineless lactobacilli.

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Effect of Diacetyl on Microorganisms.* (21054)

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It is not clear whether diacetyl enjoys a major or relatively minor role in the metabolism of plants and animals. This compound occurs in a large variety of natural products such as tobacco, butter, coffee, cocoa, beer, and honey. The citric acid-fermenting streptococci produce diacetyl and cultures of these organisms are widely used for the production of desirable flavors in dairy products(1). This compound is metabolized by bacteria, bacterial extracts and mammalian tissue through

TABLE I. Inhibitory Concentration of Diacetyl for Microorganisms.

Organism	Diacetyl (μg/ml)	Organism	Diacetyl (μg/ml)
E. floccusum	200	*A. aerogenes*	400
A. niger	"	*P. vulgaris*	"
S. carlsbergensis	"	*B. mycoides*	"
S. griseus	"	*B. subtilis*	"
E. coli	300	*B. anthracis*	"
P. pyocyaneus	"	*Cl. tetani*	"
S. paradysenteriae	"	*S. viridans*	"
S. schottmuelleri	"	*M. pyogenes*	600
S. typhosa	"	*S. hemolyticus*	800
M. tuberculosis (607)	"	*S. fecalis*	"
M. phlei	"	*S. lactis* (8043)	1200
N. catarrhalis	"	*L. casei* (7469)	1600

Laboratory strains except where indicated.

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* A portion of this work was done in the Department of Bacteriology, St. Louis University School of Medicine, St. Louis, Mo.

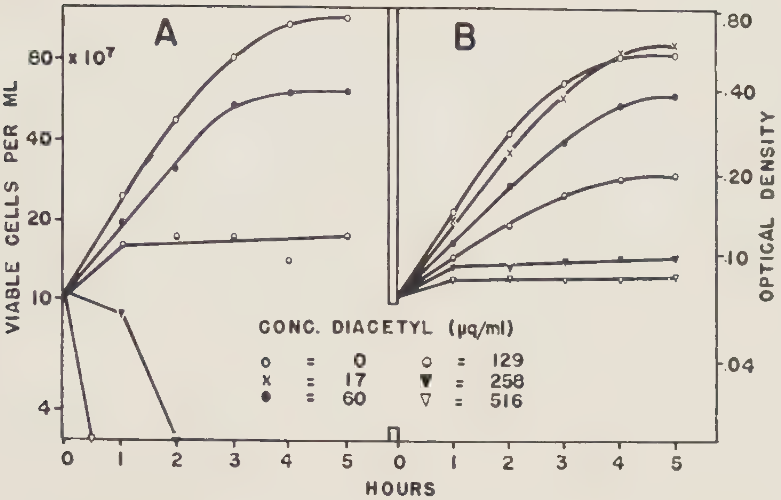

FIG. 1. Effect of diacetyl on microorganisms.

reactions in which diphosphothiamine is involved(2,3). In view of this information it seems surprising that little attention has been directed toward the antimicrobial properties of diacetyl which were first reported by O'Meara(4) and Jolander(5). The present paper describes the effect of diacetyl on the growth of a variety of microorganisms as well as a quantitative evaluation of its antibacterial action.

Experimental. The concentrations of diacetyl necessary to prevent growth of various microorganisms in Trypticase Soy broth for a 24-hour period are listed in Table I. The growth of all organisms tested was inhibited by diacetyl. Relatively small differences existed between the susceptibility of the fungi and many of the bacteria. However, the streptococci and lactobacilli, certain of which are known to produce substantial amounts of diacetyl, were the most resistant to its action. The values are maximal since an indefinite amount of diacetyl escaped from the unsealed tubes through volatilization and small amounts may have been inactivated by components of the medium. To evaluate the antibacterial action of diacetyl, *Escherichia coli* was chosen as the test organism because of its relatively simple growth requirements, its intermediate location in the diacetyl-sensitivity spectrum, and the availability of information concerning its metabolism. The medium used in this study was composed of the following ingredients per liter of solution: sodium phosphate, dibasic, 3 g; potassium phosphate, monobasic, 2 g; ammonium sulfate, 1 g; potassium nitrate, 1 g; dextrose,† 0.25 g; salts B†(6), 2.5 ml. An inoculum consisting of 6 x 10^6 organisms in the logarithmic growth phase was transferred to screw cap test tubes containing 6 ml of sterile media. Growth was followed turbidimetrically, and aliquots were plated for determination of viable cells.

Results. The rate of growth and viability of *E. coli* in the presence of varying amounts of diacetyl are shown in Fig. 1A and B. In the *logarithmic phase* the rate of growth of *E. coli* decreased progressively with increasing amounts of diacetyl. There was close agreement between viable cell counts and turbidity measurements when the concentration of diacetyl was 60 μg/ml or less. With an increase in the amount of diacetyl to 129 μg/ml a majority of the cells reproduced during the first hour only; however, a gradual increase in turbidity occurred throughout the 5-hour experiment. This increase may have been due to either slow reproduction of some cells balanced by death of others or may be explained by changes in the size of inhibited cells. A pronounced lethal action of diacetyl was effected in concentrations exceeding 129

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† Solutions sterilized by use of sintered glass filter.

TABLE II. Effect of Concentration of Medium on Action of Diacetyl.

	Optical density ($\times 10^{-3}$)								
	Log phase (2 hr)			Stationary phase (5 hr)			Final growth (22 hr)		
	Diacetyl (μg/ml)								
Conc. of medium	0	17	60	0	17	60	0	17	60
4X	243	205	163	675	550	290	820	770	460
2X	266	238	195	685	660	420	770	770	630
1X	237	231	191	490	450	415	530	695	500
½X	215	209	179	345	395	345	390	510	425

μg/ml, although the increase in turbidity during the first hour indicated that initial reproduction had occurred. Thus, diacetyl appeared to exert an inhibitory action at lower and a lethal action at higher concentrations. To test the possibility that these effects might be influenced by the initial number of cells, the amount of inoculum was varied in 5 steps from 6 x 10^5 to 12 x 10^6 cells. The rate of cellular division was constant regardless of the size of inoculum. Restoration of viability of cells(7) which had been exposed to lethal concentrations of diacetyl for 22 hours could not be demonstrated following incubation of the cells for 24 hours in buffered solutions of a variety of metabolites.

As the *stationary phase* of growth was approached the effect of diacetyl at a concentration of 17 μg/ml differed from that of higher concentrations in that turbidity finally exceeded that of the controls. This observation suggested that the normal medium was deficient in amounts of components necessary for maximal growth, and that diacetyl was serving as a substrate for growth. To test this possibility the action of diacetyl was determined in ½, 1, 2, and 4 fold (X) concentrations of the medium used previously. The results of this experiment are summarized in Table II. At concentrations of both 17 and 60 μg/ml diacetyl enhanced final growth in the most dilute medium although this effect was not observed in the more concentrated media. That a deficiency in buffering capacity and dextrose content actually existed in the 1X medium was demonstrated in experiments in which the amounts of individual components of the normal medium were increased.

The postulate that diacetyl served as a substrate for growth was confirmed by a demonstration of its disappearance from the medium during growth (Table III). Furthermore, when the *utilization per unit turbidity* (K) was calculated this was found to be greatest in deficient media. For example, in dilute medium (½X) K equaled 52 after 24 hours growth, whereas, in a more concentrated medium (2X), K equaled only 38.

Discussion. The manner in which diacetyl influences growth of *E. coli* is not understood. It has been established that the inhibitory effects of this compound remained unaltered throughout an 8-fold range of medium-concentration. Hence, inhibition may not be explained by a non-reversible combination of diacetyl with an essential substance in the growth medium. Although the possibility exists that diacetyl reacts irreversibly to inactivate essential components of the cell, failure to observe any correlation between inhibition and size of inoculum does not support this concept. The initial reproduction of cells

TABLE III. Utilization of Diacetyl by *E. coli* during Growth in ½X Medium.

Incubation, hr	Exp. conditions	Diacetyl recovered* (μg/ml) 0°C	37°C	Δ (0°-37°)	Utilization over control (Δ_B-Δ_C)
5	A. Inoc. M†	0	0	—	
	B. Inoc. M + 60 μg/ml D	57	41	16	14
	C. M + 60 μg/ml D. No inoc.	57	55	2	
21	A. Inoc. M	0	0	—	
	B. Inoc. M + 60 μg/ml D	57	24	33	22
	C. M + 60 μg/ml D. No inoc.	61	50	11	

* Diacetyl determined by method of White, *et al.*(8). Individual tubes of inoculated and uninoculated ingredients were maintained at 0°C and 37°C to control for loss of diacetyl through volatilization and possible chemical reactions.

† Inoc. M = Inoculated medium; M = Medium; D = Diacetyl.

which occurred during the first hour in the presence of inhibitory amounts of diacetyl may indicate that in addition to its utilization in a normal metabolic pathway the compound may also interfere with the metabolism of an essential substance. Studies concerning this phase of the problem are now in progress.

Summary. Diacetyl prevented the growth of all of a variety of microorganisms in a range of 200-1600 μg/ml. In quantitative studies with a single strain of *E. coli*, diacetyl exerted an inhibitory action at low concentrations (< 129 μg/ml) and lethal action at higher concentrations (> 129 μg/ml). It was demonstrated that diacetyl was utilized during growth of *E. coli* and enhanced final growth when the medium was deficient in glucose.

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Inhibitory Effect of Cortisone Hyperlipemia on Arterial Lipid Deposition in Cholesterol-Fed Rabbits.* (21055)

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Recent experiments on rabbits fed cholesterol and subjected to alloxan(1,2), detergents(3), malnutrition(4,5), and hyaluronidase(6) indicate that the amount of lipid deposited in arterial walls may be markedly at variance with the degree of hypercholesterolemia attained. In some of these experiments hypercholesterolemia was associated with increases of other serum lipid components. It has been suggested that arterial lipid deposition in cholesterol-fed rabbits is reduced if the ratio of these other lipid components, notably phospholipid, to cholesterol is increased. Administration of cortisone to cholesterol-fed rabbits, according to Oppenheim and Bruger(7), and Gordon, Kobernick, McMillan, and Duff(8), retards arterial lipid deposition. Cook *et al.*(9), however, did not find conclusive evidence of retardation of atherosclerosis in 3 cholesterol-fed rabbits that were given relatively small doses of cortisone. Kobernick and More(10) and others have observed rapid development of hyperlipemia in rabbits receiving cortisone. Rich(11) found that in cortisone hyperlipemia the increase was chiefly of fatty acids. The present experiment was designed to determine what role cortisone hyperlipemia plays in retarding arterial lipid deposition in cholesterol-fed rabbits.

Methods. Three groups, each of 6 young adult rabbits, were given daily intramuscular injections of cortisone (Cortone Acetate, Merck) and food supplements of 1% cholesterol in 3 courses according to the following scheme:

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* This investigation was supported by a research grant from the National Heart Institute of the National Institutes of Health, Public Health Service.

	Group I		Group II		Group III	
Days of exp.	Cortisone, mg	Cholesterol, %	Cortisone, mg	Cholesterol, %	Cortisone, mg	Cholesterol, %
1–14	0	0	0	1	0	1
15–28	5	0	5	1	0	1
29–42	0	0	0	0	0	0
43–56	10	0	10	1	0	1
57–70	0	0	0	0	0	0
71–84	15	0	15	1	0	1

Under this regimen the animals remained in good health throughout the experiment except for one rabbit in Group I that sickened after 8 weeks at the end of the second course of cortisone and was sacrificed along with one animal in each of Groups II and III. The *cholesterol* was dissolved in ether and mixed with rabbit food pellets. The ether was evaporated in warm air for at least several days before use. The body weights and blood pressures by ear capsule (Grant and Rothschild(12)) were recorded for one week prior to and throughout the experiment at 2- or 3-day periods. Blood (8 to 10 cc) was withdrawn by ear vein at weekly periods and analyzed for free and total cholesterol (Schoenheimer and Sperry), phospholipid (Fiske and Subbarow), proteins (Biuret), sugar (Folin and Wu). The bloods were drawn in the morning before the feed cups were replenished but the animals were not in a fasting state. Total serum lipid was determined gravimetrically on the petroleum ether soluble portion of a Bloor extract. Presence of sugar in the urine was tested for with "Clinitest" tablets (Ames Co.) on several occasions. The aortas taken at sacrifice of the animals were stained *in toto* with Sudan IV and photographed. A drawing was made of the grossly visible deposits from tracings of the photographs.

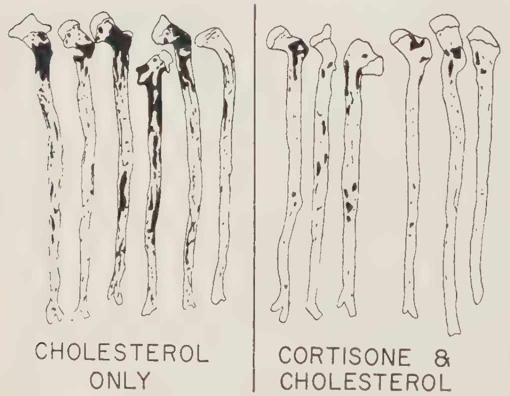

FIG. 1. Distribution of lipid deposits in aortas of cholesterol-fed and cortisone-injected rabbits. The drawings were made from tracings of photographs. The black areas represent lipid deposits. The aorta at the right of each group was from rabbits sacrificed after 8 weeks.

Results. It may be seen (Fig. 1) that lipid deposition was less than one-half as extensive in the aortas of the 6 rabbits that received both cortisone and cholesterol as in the 6 that were only fed cholesterol. Animals receiving only cortisone failed to develop any arterial lipid deposits even though there was a marked elevation in total serum lipid concentration (mean 2.64 g %) and slightly elevated blood cholesterols (mean 156 mg %).

In Table I is shown the mean concentration of cholesterol, phospholipid and total lipid at the time of sacrifice. The mean terminal concentration of cholesterol in the animals that received both cortisone and cholesterol was twice as great as in the group that was given only cholesterol even though the latter showed twice as extensive arterial lipid deposits. The total serum lipid content of the "cortisone-cholesterol" rabbits was 3 times as great as in the "cholesterol only" group. Cortisone alone caused sufficient lipemia to make rabbit serum milky after one week of daily injections of 5 mg intramuscularly. The serum of the "cortisone-cholesterol" group at the end of the experiment was almost oily as well as creamy. In both the "cortisone-cholesterol" and "cholesterol only" groups there was from 2.5 to 3 times as much cholesterol as phospholipid in the sera. Inhibition of atherosclerosis in the "cortisone-cholesterol" group cannot therefore be attributed to a lower cholesterol/phospholipid ratio. The lipids other than cholesterol and phospholipid in the sera were probably chiefly neutral fats and

TABLE I. Effect of Cortisone and Cholesterol on Blood Serum Lipids.

	Initial levels	Group I Cortisone	Group II Cortisone and cholesterol	Group III Cholesterol
		mg/% mean		
Total cholesterol	77	156	2809	1420
Phospholipid	121	355	1273	469
Total lipid	633	2640	9420	3230

fatty acids. These increased in greater proportion in the "cholesterol-cortisone" group than in the "cholesterol only" group. The amount of these fatty acids that was derived from cholesterol and phospholipid was not determined. Nevertheless the total lipid is so great compared with the other fractions involved that it is clear that the neutral fat fraction must be a major component. During the 2 periods in which cholesterol feeding was stopped the serum lipids fell rapidly but the relative proportions of the various fractions were the same as at the termination of the experiment.

Cortisone produced the same degree of hyperglycemia and glycosuria in both cholesterol-fed and non-cholesterol-fed rabbits. The mean blood sugar varied from 188 to 370 mg % depending on the dosage of cortisone and fell promptly to mean levels of from 93 to 119 mg % when cortisone administration was stopped. The urine during cortisone administration in 10 and 15 mg doses contained from traces to 4+ sugar but was occasionally negative.

The blood pressures showed no significant variations. There was a mean transient fall of 27 mm Hg shortly after cortisone injections were begun but later in the experiment cortisone injections no longer had any effect on the pressure. The blood pressures of the cholesterol-fed rabbits tended to rise from 10 to 20 mm Hg during the course of the experiment.

TABLE II. Relative Increase of Cholesterol, Phospholipid and Total Lipid in Sera of Rabbits on Cortisone and Cholesterol.

	Group I Cortisone R*	Group II Cortisone and cholesterol R*	Group III Cholesterol R*
Total cholesterol	2.0	36.5	18.4
Phospholipid	2.9	10.3	3.9
Total lipid	4.2	14.9	5.1

* Ratio, final/initial level.

The group receiving only cholesterol weighed more than the other 2 groups at the beginning of the experiment (mean 3457 g), but nevertheless gained weight consistently throughout. Their mean weight gain was 933 g. The groups receiving cortisone gained from 150 to 350 g within 24 hours each time a course of cortisone injections was begun but, consistently, this sudden weight gain was lost during the 2-week period of daily cortisone injections. It was, therefore, thought to be due to transient fluid retention. The original mean weight of the "cortisone-cholesterol" rabbits was 2804 g. Their mean weight after 10 weeks, before the third cortisone course was begun, was only 2922 g, a net gain of 116 g. The final mean weight during the third cortisone course was 3204 g. At necropsy, the cortisone injected animals had wasting of musculature and loss of adipose tissue as compared to the animals receiving only cholesterol.

The rabbits were allowed up to 200 g of food daily. No accurate measurements of intake were attempted because variable and often large amounts of food were scratched from the food cups and lost in the excreta. Nevertheless, both groups of cortisone injected rabbits consistently consumed greater quantities of food and water than did the rabbits fed cholesterol only. This may account for the greater degree of hypercholesterolemia observed in the "cholesterol-cortisone" group. Theoretically, therefore, this group should have developed greater degrees of atherosclerosis than the rabbits that received only cholesterol. The reverse was the case.

Discussion. The above findings are in accord with those of Oppenheim and Bruger(7) and very similar to those of Gordon, Kobernick, McMillan, and Duff(8). They indicate that not only alloxan but cortisone diabetes is associated with retardation of the development of atherosclerosis in cholesterol-fed rabbits. There is no evidence that any form of diabetes or Cushing's syndrome inhibits the development of atherosclerosis in man. It is becoming increasingly evident, therefore, that conditions that modify the development of atherosclerosis in cholesterol-fed animals would not necessarily have the same effect in man; they might, in fact, yield diametrically opposite results.

Nevertheless, it is important to discover why some hypercholesterolemic sera are more closely related to arterial lipid deposition than others. The role of lipids other than cholesterol in the pathogenesis of atherosclerosis is sometimes stressed. It seems remarkable, therefore, that persistent lipemias in which cholesterol does not play a dominant part do not lead to arterial deposits experimentally. In fact, the weight of evidence is in the opposite direction, namely, that when increases of serum cholesterol concentration are associated with even greater increases of other serum lipid components, the tendency for lipid to deposit in arteries is counteracted.

Gordon, Kobernick, McMillan, and Duff (8) found that cortisone reduced the degree of hypercholesterolemia and the ratio of cholesterol to phospholipid in cholesterol-fed rabbits. Our findings and those of Oppenheim and Bruger indicate that cortisone increases the severity of hypercholesterolemia and has little effect on the cholesterol/phospholipid ratio. These differences may depend upon variations in experimental procedure as Gordon *et al.*(8) pointed out. In their experiment fixed amounts of cholesterol were given daily and cortisone injections were continuous over an 8-week period. In our procedure cholesterol-containing food was supplied in larger amounts as noted above. At the end of each 2-week course of daily cortisone injections the rabbits began to lose weight and vigor but recovered during the 2-week rest periods. In any case our findings and those of Oppenheim and Bruger(7) indicate that reduction of cholesterol/phospholipid ratios is probably not the mechanism responsible for retardation of arterial lipid deposition in cortisone-treated rabbits. Our findings and those of Gordon *et al.*(8) are in accord in one particular, namely, cortisone produces a sustained alimentary type of hyperlipemia. If the inhibitory effect of cortisone on experimental atherosclerosis in rabbits depends on changes in blood lipid fractions it seems more logical to attribute this effect to the associated increase in neutral fat that accompanies the hypercholesterolemia. The possibility that cortisone inhibits arterial lipid deposition in some manner independent of its effects on blood lipids cannot be excluded. It should be noted, however, that ACTH does not produce constant or severe lipemias in rabbits(13). Oppenheim and Bruger(7) found that ACTH was less effective than cortisone in inhibiting arterial lipid deposition in cholesterol-fed rabbits. Firstbrook(4) has shown that malnourished rabbits develop less severe atherosclerosis than well-nourished ones when equal amounts of cholesterol are fed to both groups. Since rabbits receiving cortisone as well as cholesterol gained less weight than those on cholesterol alone, it is possible that malnutrition was a factor in inhibiting arterial lipid deposition in the former. McMillan, Whiteside, and Duff(5) found, however, that partly starved rabbits developed higher blood cholesterol levels than adequately fed ones when equal amounts of cholesterol were added to the food. Under these circumstances the underweight rabbits developed as much atherosclerotic change as the well nourished ones with lower blood cholesterol concentrations. The rabbits receiving both cortisone and cholesterol are more comparable to the partly starved ones of McMillan, Whiteside, and Duff(5) since they, too, had much higher blood levels than the rabbits that received only cholesterol. Since, in spite of this, the degree of arterial lipid deposition observed in the cortisone-treated animals was decidedly less than in the controls, it may be concluded that this difference in severity was probably not entirely the result of malnutrition. The variations in blood pressure observed were not sufficiently large to have been a factor in the inhibition of lipid deposition in the cortisone-treated rabbits.

Summary. Administration of cortisone to rabbits caused hyperlipemia due chiefly to an increase of lipids other than cholesterol and phospholipid. Cholesterol-fed rabbits treated with cortisone developed severer degrees of hypercholesterolemia and hyperlipemia than rabbits on cholesterol alone. The relative proportion of cholesterol in the serum lipids of cholesterol-fed rabbits on cortisone was less than in the serum lipids of rabbits on cholesterol alone. Significantly less lipid was deposited in the arteries of the cortisone-treated, cholesterol-fed animals than in the cholesterol-fed controls. It is suggested that hyperlipemias in which the lipid fractions other than cholesterol undergo the greatest increase, are less likely to be associated with arterial lipid deposition than hyperlipemic states in which cholesterol is the major constituent.

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Copper Metabolism. XII. Influence of Manganese on Metabolism of Copper.* (21056)

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The clinical and pathological similarity between the manifestations of chronic manganese poisoning in man and the changes which occur in hepatolenticular degeneration has been noted several times(1-3). Hepatolenticular degeneration is now known to be characterized by aminoaciduria, hypocupremia, hypercupriuria and an increase in the amount of copper in the tissues(4-9). Studies on the metabolism of copper in man or in animals with manganese toxicity have not been reported, however.

It was, therefore, considered to be of interest to determine whether or not the administration of large amounts of manganese could be shown to influence the metabolism of copper in animals. In this report observations are presented on the level of copper in the plasma, urine and tissues of rats given large amounts of manganese. The influence of manganese on the red blood cells, the urinary excretion of amino acids and the content of iron in the plasma and tissues were also studied. A preliminary report of this work has been published(10).

Methods. Eighty-three male weanling rats of the Sprague-Dawley strain were divided into 4 groups (Table I), housed in individual

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TABLE I. Observations on Copper and Iron Content of Various Tissues and on Urinary Excretion of Amino Acids. Ten animals in each group, sacrificed at 86 days.

	A Control	B High Mn	C High Cu	D High Mn + Cu
Body wt, g	371 ± 9.1	171 ± 7.2	333 ± 0.9	125 ± 5.0
Plasma copper, μg %	107 ± 3.6	142 ± 3.9	126 ± 3.6	194 ± 11.7
Urine ” * μg/day	1.8	1.0	3.1	1.8
Liver ” μg/g	5 ± 0.2	5 ± 0.2	39 ± 14.1	670 ± 66.4
μg/rat	69 ± 3.1	29 ± 1.3	457 ± 161.4	3070 ± 276.6
Kidney ” μg/g	8 ± 0.6	5 ± 0.2	23 ± 3.9	55 ± 23.4
μg/rat	19 ± 1.7	7 ± 0.2	51 ± 7.0	71 ± 29.1
Brain ” μg/g	3.6 ± 0.21	4.4 ± 0.15	3.9 ± 0.10	5.0 ± 0.21
μg/rat	6.6 ± 0.25	7.3 ± 0.22	6.8 ± 0.11	8.0 ± 0.24
Plasma iron,* μg %	301	112	265	128
Liver ” μg/g	160 ± 11.0	40 ± 2.2	130 ± 5.0	40 ± 2.0
μg/rat	2120 ± 198.0	200 ± 3.2	1620 ± 67.7	200 ± 11.1
Kidney ” μg/g	78 ± 2.8	32 ± 2.2	75 ± 2.8	41 ± 4.1
μg/rat	194 ± 11.5	47 ± 3.6	161 ± 6.4	54 ± 4.8
Urine amino N,* mg/day	1.4	0.8	1.2	0.6

* Value obtained from pooled specimens.
Figures refer to mean ± stand. error.

cages, and fed the following basal diet *ad libitum*: purified casein, 20%; sucrose, 64%; lard, 11%; and salt mix(11), 5%. Vitamins were added to the basal diet as follows (mg/kg of diet): thiamin hydrochloride, 12.5; riboflavin, 6; nicotinic acid, 60; pyridoxine hydrochloride, 10; calcium pantothenate, 25; inositol, 5; para-aminobenzoic acid, 5; pteroylglutamic acid, 15; vit. E, 7.6; and vit. K, 7.6. Vit. A in an amount of 23000 units and vit. D in an amount of 4250 units per kilogram of diet were also added.

The general design of the experiments and the schedule of the intraperitoneal injections are recorded in Fig. 1 together with the growth curves. All animals in groups A, B, and C survived the duration of the experiment. Three animals in group D died between the 68th and 83rd days. The remaining animals in this group were thin, inactive, and failed to gain weight. Therefore, on the 86th day 10 animals in each group were anaesthetized with ether, phlebotomized by cardiac puncture and the organs removed for chemical analysis. The remaining animals continued to survive and on the 120th day of the experiment they were sacrificed by ether anaesthesia. Total body copper and iron analyses were performed on these rats. *Hemoglobin* was determined by the oxyhemoglobin method in the Evelyn photoelectric colorimeter. The other hematologic determinations were performed as outlined by Wintrobe(12). The method employed for the measurement of plasma copper has been described by Gubler, *et al.*(13); for plasma iron by Hamilton *et al.*(14); for tissue iron by Gubler *et al.*(15); and for total body copper and iron by Chase *et al.*(16). A modification(17,18) of the method of Folin was used for the determination of urinary alpha amino-nitrogen. Twenty-four-hour urine samples were collected from ten rats on the 84th day of the experiment. The samples were pooled and used for the determination of both alpha amino-nitrogen and copper(19).

Results. The administration of manganese alone was associated with a significant increase (P <0.01) in the concentration of copper in the plasma and brain and a decrease in the urinary excretion of copper (Table I). The concentration of copper in the liver was not significantly influenced, and the concentration of copper in the kidneys was decreased. The administration of large amounts of copper was associated with a significant increase (P <0.01) in the concentration of copper in the plasma, urine, liver, and kidneys but not in the brain. When large amounts of both copper and manganese were given (group D)

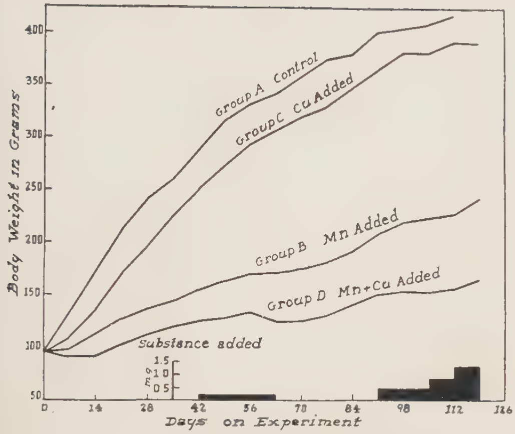

FIG. 1. Influence of manganese, copper, and copper in addition to manganese on growth of rats. All animals received the basal diet and the following supplements: Group A, none; group B, 4% manganese chloride; group C, 0.1% copper sulfate; group D, 4% manganese chloride and 0.1% copper sulfate. In addition, the animals in group B were given manganese chloride ($MnCl_2 \cdot 4H_2O$) intraperitoneally in amounts indicated; those in group C were given ''Cupralene'' in this way and those in group D both manganese chloride and ''Cupralene.'' These compounds were injected 3 times weekly. Half the animals in each group were sacrificed at 86 days, the remainder at 120 days. As a result those sacrificed at 86 days received a total of 4.5 mg $MnCl_2$ intraperitoneally while the remainder received a total of 25.5 mg. The total amounts of ''Cupralene'' received by the animals sacrificed at these different times were also 4.5 and 25.5 mg, respectively.

the concentration of copper in the plasma, liver, kidneys, and brain was significantly increased ($P < 0.01$) as compared with the values in the animals given the same amount of supplementary copper (group C). A decreased urinary excretion of copper was associated with this retention of copper in the tissues.

The concentration of iron in the plasma, liver and kidneys was significantly lower ($P < 0.01$) in the animals given manganese, either alone (group B) or with copper (group D). Aminoaciduria was not observed in any of the 3 experimental groups.

As shown in Table II, the administration of manganese alone (group B) had little influence on total body copper; the administration of large amounts of copper (group C) caused an increase in total body copper. When large quantities of both copper and manganese (group D) were given simultaneously, the total body copper was approximately twice that found in the animals receiving the same amount of copper without added manganese. Since these animals were considerably smaller than the animals in group C, the amount of copper expressed in terms of $\mu g/100$ g of body weight was increased about 5-fold.

The administration of manganese alone (group B) was associated with a significant decrease ($P < 0.01$) in the total amount of iron in the body and a significant decrease ($P < 0.01$) in the amount of iron per 100 g of body weight. When supplementary copper was given in addition to manganese, the decrease in iron (mg/100 g of body weight) was partially overcome, but the total body iron was less than in the animals in group B. The administration of copper alone (group C compared with group A) had no significant influence on total body iron.

The hematologic observations are summarized in Table III). The animals receiving large amounts of manganese (group B) developed a mild anemia which was microcytic and slightly hypochromic and was accompanied by a slight increase in reticulocytes. The animals given large quantities of copper (group C) failed to develop anemia or morphologic alterations in the erythrocytes. When large amounts of copper were given in addition to manganese (group D) the hematologic changes observed were similar to those which occurred when large amounts of manganese alone were given.

Discussion. From the data presented it is evident that in the presence of a large amount of dietary copper, the administration of large quantities of manganese is associated with a marked increase in the retention of copper in the body, particularly in the liver and spleen but also in the plasma and brain.

The administration of large quantities of manganese, either with or without the administration of excessive amounts of copper was associated with the development of microcytic, hypochromic anemia. The concentration of iron in the body of the rats fed large amounts of manganese was significantly reduced, particularly in the liver. Therefore, it is quite possible that the anemia was the result of a mild deficiency of iron. However,

TABLE II. Total Body Copper and Iron. Ten animals in each group, sacrificed at 120 days.

	A Control	B High Mn	C High Cu	D High Mn + Cu
Body wt, g	419 ± 10.9	234 ± 12.1	392 ± 7.8	166 ± 8.7
" copper, μg/100 g	126 ± 3.5	193 ± 8.9	470 ± 31.3	2319 ±378.3
μg/rat	572 ± 16.4	445 ± 18.5	1833 ±115.0	3741 ±537.0
" iron, mg/100 g	4.72± 0.177	3.62± 0.247	4.81± 0.108	4.32± 0.193
mg/rat	19.6 ± 0.71	8.6 ± 0.85	18.8 ± 0.38	7.1 ± 0.11

Values represent mean ± stand. error.

the degree of reduction in the plasma iron level and in the total body iron would not seem to be sufficient to account for the development of anemia on this basis. It should be noted that the anemia associated with an excessive intake of manganese is also morphologically similar to the anemia which appears in swine deficient in copper(20). It is possible that manganese forms a complex with copper thus making the copper physiologically unavailable or that the manganese in some manner blocks the action of copper-containing enzymes which are essential for normal erythropoiesis. Such an interaction has been postulated by Marston(21) to account for the observation that moderate amounts of molybdenum, administered to rats given rations which supply sufficient copper to provide for maximal growth and erythropoiesis, will increase materially the copper concentration in the tissues and precipitate the symptoms of copper deficiency.

In regard to the possible role of manganese and copper in the pathogenesis of hepatolenticular degeneration, it is worthy of note that, although in our experimental animals the tissue levels of copper were increased to a degree comparable to that observed in patients with hepatolenticular degeneration(8), a syndrome simulating this disease was not produced. Neurologic abnormalities, corneal pigmentation, aminoaciduria, and histologic alterations[†] in the brain were not observed. Furthermore, the plasma copper level was increased rather than decreased, and the administration of manganese was associated with a decrease rather than an increase in the excretion of copper in the urine. In the animals receiving excessive manganese (group B), copper (group C), and copper and manganese together (group D) acute widespread necrosis of the liver with varying degrees of regeneration of liver parenchyma was observed.[†] However, increase in fibrous tissue, such as is found in the livers of patients with hepatolenticular degeneration was not present. It cannot, of course, be concluded from these relatively short term studies in rats that copper and manganese either do or do not play a role in the pathogenesis of the lesions observed in hepatolenticular degeneration.

Summary. 1. Administration of large amounts of manganese to rats was associated with an increase in the concentration of copper in plasma and brain, a decrease in the urinary excretion of copper, a decrease in the concentration of copper in the kidneys, no alteration in the concentration of copper in the liver, and microcytic, hypochromic anemia. The total amount of copper in the body was not increased. 2. Administration of large amounts of copper was accompanied by an increase in total body copper and an increase in the concentration of copper in the plasma, urine, liver; and kidneys but not in the brain. Anemia did not occur. 3. Simultaneous administration of large amounts of both manganese and copper resulted in a marked increase in total body copper, an increase in the concentration of copper in the plasma, liver, kidneys and brain, and microcytic, hypochromic anemia. The total amount of copper in the body of these animals was twice as great as in rats given the same amount of supplemental copper alone. The concentration of copper (μg/100 g of body weight) was increased 5-fold. 4. It is suggested that manganese may form a complex with copper which makes the latter unavailable or that in some manner it blocks the action of copper-containing enzymes.

[†] E. J. Eichwald, unpublished observations.

TABLE III. Hematologic Studies. Ten animals in each group.

	A	B	C	D
	Control	High Mn	High Cu	High Mn + Cu
Red cell count × 10^6/mm^3	8.91 ± .323	9.40 ± .192	8.66 ± .238	10.06 ± .334
Hemoglobin, g %	17.5 ± .19	11.7 ± .64	17.6 ± .32	12.8 ± .75
Vol packed red cells, ml/100 ml	51 ± .8	37 ±1.3	49 ± .7	40 ±1.9
Mean corpuscular vol, cμ	57 ±1.7	40 ±1.8	58 ±1.3	40 ±2.7
Mean corpuscular hemoglobin, μμg	20 ± .6	12 ± .5	21 ± .3	13 ± .9
Mean corpuscular hemoglobin conc., %	35 ± .4	32 ±1.0	36 ± .2	32 ±1.2
Reticulocytes, %	2.6 ± .23	5.7 ± .57	2.7 ± .20	6.8 ±1.19

Values represent mean ± stand. error.

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Destruction of I^{131} Labeled Insulin by Liver Slices.* (21057)

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The biological activity of insulin is destroyed after incubation with homogenates, extracts and slices of liver(1,2). Two systems appear to be involved in the process, an insulin-inactivating system which has the properties of an enzyme (insulinase)(1) and an inhibitor of this system which appears to be a heat-stable, dialyzable non-protein factor (insulinase-inhibitor)(3). The mode of action or the specificity of insulinase has not been established principally because of the difficulties involved in the biological assay of small amounts of insulin.

With the availability of I^{131} it has become possible to label insulin and to trace the fate of the insulin molecule *in vivo* and *in vitro* (4,5). Since the insulin-inactivating activity of liver slices represents the balance between the actions of insulinase and of insulinase-inhibitor(2) the effect of such slices on the *in vitro* degradation of labeled insulin (I^{131} insulin) was studied.

Methods. Fed male rats of from 150 to 200 g in weight were decapitated and as much blood as possible was drained from the carcass of each animal. The liver was removed and placed in cold phosphate buffer, pH 7.4. Slices of approximately 1 cm² in size were prepared with a Stadie-Riggs apparatus(6). After blotting with filter paper, the slices were weighed and the desired amount placed into a flask containing 2 ml of the phosphate buffer. Various concentrations of I^{131} insulin (or I^{131} human serum albumin) in 1 ml buffer were added to the flasks which were shaken in a Dubnoff incubator at a rate of 70 cycles/minute and maintained at 37°C. At the end of the incubation period, an aliquot of the incubation mixture was precipitated with an equal volume of 10% trichloracetic acid. The supernatant and precipitate were separated by centrifugation and after several washings with buffer the radioactivity of the precipitate and of the supernatant plus washings was measured in a well-type gamma counter. Each count was corrected for background and coincidence and the amount of I^{131} insulin degraded was calculated from the percentage of the total counts of the precipitate plus supernatant that was found in the non-protein fraction. In some instances, in addition to the radioactivity measurements, the hypoglycemic activity of an appropriate aliquot of the incubation mixture was determined in rabbits and expressed as the 'pooled percentage of the initial blood sugar level'. An increase in the 'pooled percentage' over that produced by a similar quantity of insulin which has not been incubated serves as an index of insulinase activity(1). Each experiment was performed in triplicate. I^{131} insulin was prepared by subjecting crystalline insulin,† which assayed 25 units per mg to the procedure described by Talmage, Dixon, Bukantz and Dammin(7). From 98 to 99% of the I^{131} insulin was precipitable with 10% trichloracetic acid. I^{131} human serum albumin was prepared in the same manner. The insulinase-inhibitor was prepared from liver by the procedure described in a previous communication(3). Blood sugar was determined by Nelson's method(8).

Results. Incubation of I^{131} insulin with liver slices results in a concomitant reduction in the biological activity of insulin (*i.e.*, an increase in the 'pooled percentage') and a degradation of the insulin molecule (*i.e.*, an increase in the radioactivity of the non-protein fraction of the incubation mixture) (Fig. 1). Accordingly, the insulinase activity of liver slices is associated with a degradation of the insulin molecule.

The relation between the degradation of I^{131} insulin and the length of incubation was

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† We are indebted to Eli Lilly Co. for generous supplies of crystalline insulin. The I^{131} was made available by the Addison H. Gibson Laboratory.

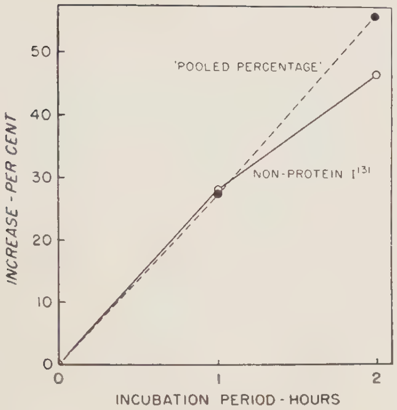

FIG. 1. Inactivation and degradation of I^{131} insulin by liver slices. Each point represents 3 sets of 500 mg liver slices incubated with 3 units of I^{131} insulin at 37°C and pH 7.4 for the designated time.

determined by keeping the quantity of liver slices constant (approximately 500 mg wet weight) and varying the period of incubation. It is apparent from Fig. 2 that the degradation follows zero order kinetics for at least during the first 30 minutes. Thereafter, the reaction follows first order kinetics.

The relation between the quantity of liver and the degradation of insulin was determined by incubating I^{131} insulin with from 1 to 6 slices for 30 minutes. As has been demonstrated previously for insulinase activity(2), the greater the quantity of liver slices, the greater is the degradation of the I^{131} insulin (Fig. 3).

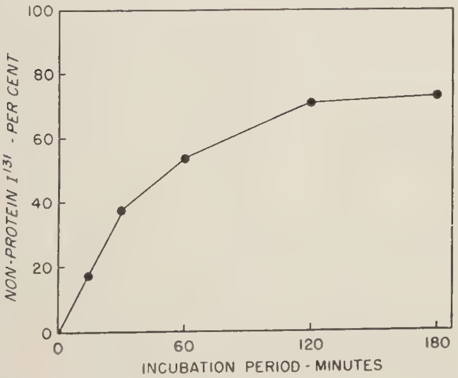

FIG. 2. Relation between length of incubation and degradation of I^{131} insulin. Each point represents mean effect of 3 sets of 500 mg liver slices incubated with 3 units I^{131} insulin at 37°C and pH 7.4 for the designated time.

That the degradation of I^{131} insulin follows first order reaction kinetics is apparent also when the quantity of liver slices are kept constant and the concentration of I^{131} insulin substrate is varied (Fig. 4). The Km of the degradation produced by liver slices from different rats varied markedly. That this variation may be due to the presence of variable quantities of endogenous insulinase-inhibitor in the liver slices is suggested by the effect of exogenous insulinase-inhibitor on the activity of liver slices incubated with varying concentrations of I^{131} insulin (Fig. 5). The Lineweaver-Burk plot of the data in Fig. 5 suggests that the inhibitor is a non-competitive inhibitor(9).

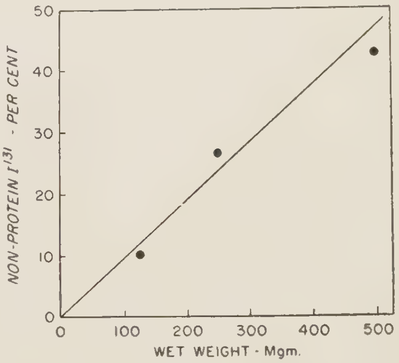

FIG. 3. Relation between quantity of liver and degradation of I^{131} insulin. Each point represents mean effect of 3 sets of liver slices of specified wet weight incubated with 3 units I^{131} insulin for 30 min. at 37°C and pH 7.4.

As a step towards establishing the specificity of the enzyme involved in the degradation of insulin, liver slices were incubated with I^{131} human serum albumin. Negligible amounts of the albumin were degraded during a 3-hour period of incubation while a marked degradation of a similar quantity of I^{131} insulin occurred during the same interval on incubation with similar liver slices.

Comment. The data indicate that relatively intact cells of surviving liver slices are capable not only of inactivating the biological activity of insulin but also of degrading the insulin molecule. If both phenomena are due to a single enzyme system then the rate of degradation of I^{131} insulin can serve as a measure of insulinase activity. The inhibition of both

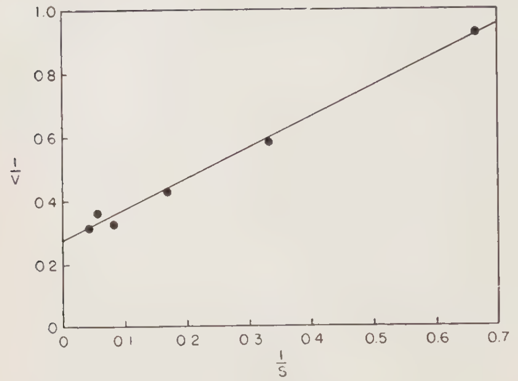

FIG. 4. Effect of concentration of I^{131} insulin on its degradation by liver slices. Each point represents mean effect of 3 sets of 500 mg of liver slices incubated at 37°C and pH 7.4 for 30 min. (V) with different concentrations of I^{131} insulin (S).

insulinase activity and of the degradation of I^{131} insulin by the insulinase-inhibitor supports the possibility that the two phenomena are intimately related and due to an enzyme system which follows first order kinetics. That the enzyme system involved is not some non-specific proteolytic system is suggested by the inability of liver slices to degrade I^{131} human serum albumin.

The measurement of insulinase activity by the biological technic(1) does not permit the detection of small quantities. With the degradation of I^{131} insulin as an index of insulinase, it is possible to detect relatively minute quantities of the enzyme in blood and other tissues.

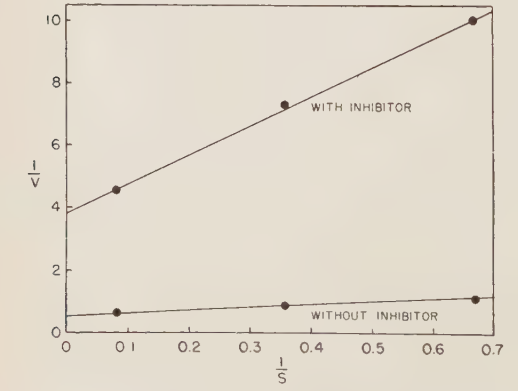

FIG. 5. Effect of insulinase-inhibitor on degradation of I^{131} insulin by liver slices. Each point represents mean effect of 3 sets of 500 mg of liver slices incubated at 37°C and pH 7.4 for 30 min. (V) with different concentrations of I^{131} insulin (S). 0.5 ml of insulinase-inhibitor was added to incubation mixture of one group of liver slices.

There is little doubt that diabetes mellitus in man is due to an insufficiency of insulin relative to the requirements by the tissues. In only a small proportion of instances, however, can the metabolic derangement in man be attributed solely to a diminution in the production of insulin(10). The demonstration that insulin can be destroyed by slices of liver lends support to the hypothesis that the insulin insufficiency of the patient with diabetes mellitus may be due to an increase in the rate of insulin destruction consequent to an increase in insulinase activity or a decrease in the availability of the insulinase-inhibitor(11).

Summary. 1. Insulin labeled with I^{131} was used to determine the effect of liver slices on the degradation of insulin. 2. The insulin-inactivating activity of slices of rat liver (insulinase) is associated with a degradation of I^{131} insulin. 3. The degradation of I^{131} insulin by liver slices follows first order kinetics and is related directly to the quantity of liver slices employed. 4. A non-protein fraction of liver (insulinase-inhibitor) which inhibits the action of insulinase also inhibits the degradation of I^{131} insulin by liver slices.

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Prevention of Bone-Marrow Aplasia Induced by Aminopterin with Folacin and Folinic Acid. (21058)

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The prevention of aminopterin toxicity in the rat, chick, mouse and bacteria has been extensively studied(1-3). As judged by blood picture, weight responses and survival, there appears to be general agreement that folinic acid is far more effective than folacin in overcoming the toxic effects of aminopterin in rats and mice.

During the course of studies on chemically induced bone marrow aplasia, the marked inhibitory effect of aminopterin on rat bone marrow(4,5) was confirmed. This paper presents data showing the effects of the simultaneous administration of aminopterin and folacin type compounds on the cellular elements of rat bone marrow. The procedure followed offers a convenient method for assay of compounds effective against aminopterin. Under these conditions the effects of several pterin compounds of variously reported biological activity have been recorded.

Experimental. Weanling albino rats, reared in our own laboratory were given a diet of the following composition: Labco casein, 18; Cerelose, 73; Wesson Oil, 3; cod liver oil, 2. The following amounts of B vitamins were given as an oral supplement daily: 100 μg thiamine mononitrate, pyridoxin • HCl and nicotinamide, 200 μg Ca pantothenate and riboflavin and 10 mg choline chloride. Ten μg aminopterin was given orally in 0.05 ml or 0.1 ml volume. Immediately after administration of the inhibitor the compound under study was administered as an intramuscular injection. At the end of 5 days surviving animals were killed and femur bone marrow removed and sectioned as described below. The principle upon which the evaluation of bone marrow was based was the degree of injury to development of hematopoietic elements. The criteria for scoring are summarized in Table I.

TABLE I. Evaluation of Injury to Bone Marrow.*

Description	Degree of injury	Score
Normal cellularity	0	0
Foci of diminished cellularity between areas of apparently normal hematopoietic tissue	+	1
Marked general diminution of cellularity; yet all hematopoietic elements present	++	2
Far-going destruction with islets of preserved hematopoietic elements	+++	3
Complete absence of hematopoietic elements	++++	4

Intermediary indices:
0/+ = ½, +/++ = 1½, ++/+++ = 2½, +++/++++ = 3½.

* Sections through the largest plane of pencil of femoral bone marrow. Fixative: 10% formaldehyde, 90% ethanol. Stain: hematoxylin-eosin. Magnification, 200 ×.

Results. The administration of 10 μg aminopterin to weanling rats for 5 days resulted in a score of about 3.0. The effects of simultaneous injection of folacin or folinic acid* (leucovorin) are shown in Table II. That leucovorin was much more effective in preventing the toxic manifestations due to aminopterin is clearly shown. Under the acute conditions of these experiments, the administration of 5 mg folacin was not as effective as 150 μg leucovorin in preventing the toxic effects of 10 μg aminopterin.

The effects of 5 mg pteroyltriglutamic acid, 1 mg N-10-nitroso pteroylglutamic acid, and 1 mg N-10-formyl pteroylglutamic acid were also studied. The data show no evidence of preventive activity.

It was also of interest to investigate the influence of ascorbic acid and/or vit. B_{12} on the response to sub-optimal amounts of folacin. Under the conditions of these experiments, no influence by these 2 compounds

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* Folinic acid administered as synthetic Ca Leucovorin.

TABLE II. Prevention of Aminopterin Induced Bone-Marrow Aplasia by Leucovorin and Folacin.

Compound administered*	No. rats	Score‡
None†	94	2.9
Folacin, 20 μg-1 mg	67	2.7
2 mg	24	1.9
3	15	1.3
4	5	1.1
5	15	.5
Leucovorin, 10 μg	20	2.1
20	10	.7
50	10	.3
100	43	.3
125	20	.2
150	5	.0

* All rats received 10 μg aminopterin/day orally. Folic acid and leucovorin administered intramusc.

† These rats were untreated controls, concurrent with those in the table.

‡ Avg values.

when administered alone or in combination with folic acid could be demonstrated.

Discussion. The data presented indicate that leucovorin is far more effective than folacin in preventing toxic effects of aminopterin in the rat. Thus, the evidence from studies on bone marrow is in qualitative agreement with that obtained when growth and survival are utilized as standards of comparison. Sauberlich has reported that leucovorin appeared to be 10 times more effective than folacin when fed in the diet(3). In experiments with mice, Broquist and coworkers (2) have reported that an amount of leucovorin equal to that of administered aminopterin was required to reverse the toxicity of aminopterin when the 2 substances were injected simultaneously. Under the conditions reported in this paper leucovorin appears to be about 50 times more active than folacin in preventing hematopoietic changes in the marrow. Furthermore, approximately 150 μg leucovorin had to be administered to prevent the toxicity of 10 μg aminopterin.

The failure of pteroyl tri-glutamic acid to prevent the aminopterin toxicity even at a level of 5 mg per rat per day is interesting. The tri-glutamate has been reported to replace folacin for chick growth(6) and also to produce hemopoietic responses in pernicious anemia and other megaloblastic anemias(7-9). It is possible that an even larger dose than 5 mg is required to prevent the acute aminopterin effect. This is now under investigation. On the other hand, in view of the lack of effect of a comparable amount of folacin, it is not surprising that the N-10-formyl and N-10-nitroso derivatives, both of which have been reported to possess folacin activity in the chick(6), were without effect under the conditions of these experiments.

Various reports have indicated that ascorbic acid may be involved in the conversion of folacin to folinic acid(10-12). In the present study, however, ascorbic acid administration neither prevented the toxic effects of aminopterin nor influenced the response obtained with folacin. The simultaneous administration (orally or intramuscularly) of ascorbic acid and/or vit. B_{12} with sub-maximum (Table I) amounts of folacin was without effect.

Summary and conclusions. 1. The daily oral administration of 10 μg aminopterin to weanling rats induced a severe aplasia in the bone marrow within 5 days. 2. The simultaneous injection of 150 μg leucovorin completely prevented the aminopterin toxicity. 3. Simultaneous injection of folacin was also effective in preventing toxic effects of aminopterin. Folacin was about 1/50th as active as leucovorin. 4. Ascorbic acid and/or vit. B_{12} did not influence the effect of folacin, *i.e.*, no augmentation of submaximum amounts of folacin by either ascorbic acid and/or vit. B_{12} was observed.

We are indebted to Miss H. Ritter, Mrs. Marcia Walker and Mrs. Elizabeth Ross for the preparation of the slides. The samples of pteroyl triglutamic acid, N-10-formyl pteroylglutamic acid and N-10-nitroso-pteroylglutamic acid were supplied by Dr. Robert Angier of these laboratories.

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Effect of Muscular Exercise on Circulating Thyroid Hormone.* (21059)

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Recent studies by many investigators have dealt with the effect of various injurious agents and stimuli on the uptake of I^{131} by the thyroid gland. For example, Bogoroch and Timiras(1) found a consistent decrease in the uptake of I^{131} by the thyroid glands of rats after the injection of formalin or transection of the spinal cord. They also studied the effect of forced muscular exercise on the uptake and found that in one group there was no significant change, while in a second group there was a significant depression. Increased secretion of thyroid hormone on exposure to cold has been demonstrated in rats by Dempsey and Astwood(2) and increased peripheral utilization of the hormone in rats by Rand(3) and by Bondy(4), and in man by Ingbar and Kleeman(5). Bondy also found in rats that 2 hours of swimming caused a pronounced fall in serum protein bound iodine.

The present study was undertaken to determine the effect of muscular exercise in man on the level of circulating hormone and on the rate of disappearance of injected l-thyroxine labeled with radioactive iodine.

Procedure. 1) *Moderate exercise.* One female and 5 male medical students and physicians in good health varying in age from 20 to 36 years walked 14 miles in 4 to 4½ hours. In the first 2 subjects the serum butanol extractable iodine (BEI) concentration was determined at the start, after 7 miles, at the end of the walk, and 5 hours and 16 hours after the end of the walk. Control BEI determinations were made on the same subjects at the same hours during an average day. The second 2 subjects received an injection of 40 μc of radio-thyroxine 5 days before the walk. Serum radioactivity was determined at 9 a.m., 12 N., and 5 p.m. daily for 16 days. On the day of exercise the serum radioactivity was estimated and BEI's determined at the start, at the end of 7 miles, at the end of 14 miles and the following day. The third 2 subjects walked the same distance and BEI determinations performed at the beginning and end of the exercise and on the following morning. 2) *Severe brief exercise.* Six healthy male subjects swam at maximum speed for 15 minutes. All were good swimmers but had not swum for several months and were acutely exhausted at the end. Serum protein bound iodines were estimated before and at the end of the swimming.

Methods. 1) Butanol extractable iodine was measured by the method of Man(6). 2) The radio-thyroxine disappearance curves were performed according to the method of Sterling(7), the subjects being given saturated solution of potassium iodide to minimize re-

TABLE I. Serum BEI's of Subjects Walking 14 Miles (γ %).

Subject	B.	L.	S.	J.	H.	T.
Start	6.8	5.9	4.1	4.7	5.0	4.8
Midpoint, 2 hr	6.4	5.3	4.0	5.2		
End	6.5	5.6	4.4	3.5	4.9	5.2
5 hr after exercise	6.9	6.0				
Next a.m.	6.4	5.9	3.3	4.3	5.9	4.7
△ end-start	−.3	−.3	+.3	−1.2	−.1	+.4

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* Supported in part by a Grant from the U. S. Public Health Service.

† Work done during tenure of Postdoctoral Fellowship of National Foundation for Infantile Paralysis.

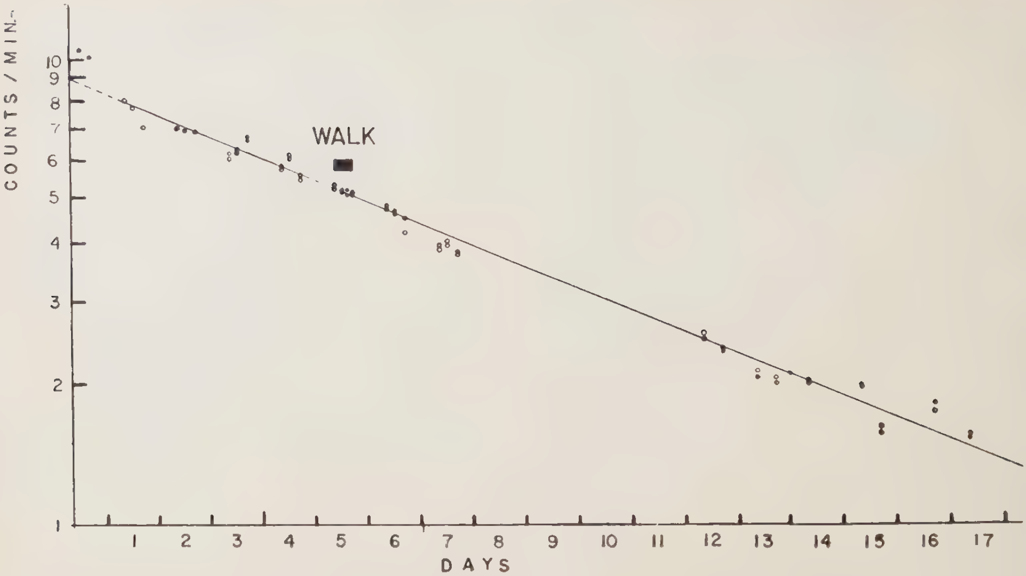

FIG. 1. Radio-thyroxine disappearance curve of subject J. Abscissa = days after injection of radio-thyroxine. Ordinate = counts per minute per cc of plasma.

utilization of radioiodide liberated from thyroxine breakdown. 3) The alkaline ash method of Barker, Humphrey and Soley(8) was used for the determination of protein-bound iodine.

Results. The results obtained in the 6 subjects who walked 14 miles are given in Table I. The reproducibility of the BEI method has been statistically analyzed. For a range of 2 to 8 γ % the difference between the means of two sets of duplicates must be 1 γ % to indicate a significant change ($p = 0.01$). Only one subject, J., showed a significant fall after exercise; but this concentration was not significantly below the BEI determined the following a.m. The BEI failed to change during an average day in the first 2 subjects.

The normal slope of the thyroxine degradation curve showed no change on the day of exercise in either subject. Fig. 1 represents the thyroxine degradation curve of one of the 2 subjects given radio-thyroxine.

In Table II the protein bound iodines before and after swimming are presented. No significant change was observed.

Blood hemoglobin and hematocrit and serum protein concentration were estimated in some of the subjects to rule out any change due to hemodilution or hemoconcentration. No significant change was found.

Discussion. The conception has been entertained that acute metabolic demands of the body may increase the peripheral utilization of thyroid hormone. In the present study muscular exercise resulted in no evident change in the concentration of circulating thyroid hormone. The stable hormone concentration could not be attributed to increased secretion counterbalancing increased removal from the blood in view of the unaltered radio-thyroxine disappearance curves. Within 2 days of injection of radio-thyroxine the decrease in serum radioactivity formed a straight line on semilogarithmic plot representing the rate of degradation of the hormone(7). With the method used a measurable change in the slope of the disappearance curve should be produced by about a 20% increase in the rate of hormone degradation on the day of exercise.

TABLE II. Serum PBI's of Subjects Swimming 15 Minutes (γ %).

Subject	A	B	C	D	E	F
Start	7.0	7.5	6.5	7.7	7.2	6.5
End	6.5	8.4	8.4	6.8	5.4	6.1
△ end-start	–.5	+.9	+1.9	–.9	–1.8	–.4

The fact that in the present study no change in thyroid metabolism occurred may indicate that a much more prolonged change in the metabolic needs of the body is required in order to alter thyroid function. Previous studies on cold exposure have demonstrated increased rate of removal of circulating thyroid hormone. However, maintenance of body temperature is a major function of thyroxine. Need for the calorigenic action of the hormone on exposure to cold may prove to be the most consistent means of increasing the utilization of thyroxine. In the rat experiment of Bondy and Hagewood(4) the temperature of the water (27°C), rather than the muscular exercise, may have been enough to account for the fall in protein bound iodine. Tan *et al.* have shown that the survival time of rats swimming in water which is below body temperature is limited by the ability of the rat to maintain body temperature at its normal level(9). Muscular exercise itself increases heat production and this may militate against increasing the rate of utilization of thyroid hormone in the present experiments by increasing the body temperature. Whether or not stimuli other than cold do affect the rate of the peripheral metabolism of thyroid hormone remains to be determined. Previous studies of the effect of injurious agents have dealt mainly with the thyroidal uptake of I^{131} rather than with the rate of disappearance of circulating hormone. The uptake of I^{131} may be influenced by many factors other than the rate of utilization of thyroxine, such as previous diet, adrenal cortical, pituitary and renal function.

Summary. Muscular exercise in human subjects produced no alteration in peripheral utilization of thyroid hormone as measured by concentration of circulating hormone and by rate of disappearance of injected radiothyroxine.

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Effect of ACTH on Strength of Intestinal Anastomoses in Dogs. (21060)

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Since the original observations by Ragan (1) concerning the retardation of wound healing in patients by ACTH, several studies have been done dealing with this phenomenon. The investigations of Ragan(2), Spain(3), and others in rabbits and mice indicate that ACTH retards the healing of wounds. Recent experiments on skin wounds in guinea pigs(4) and rats(5), and on skin wounds and cardiovascular suture lines in dogs(6,7) failed to demonstrate significant differences in the healing of wounds of control animals and those treated with ACTH or cortisone. The conflict in these experimental results may be attributable to differences in the species of experimental animal, the particular tissue studied, or to the dosage of the hormone used.

The effect of ACTH and cortisone on anastomoses in the gastrointestinal tract is important for obvious reasons. Great caution has been advocated in the use of these agents in the presence of healing anastomoses(8). In one clinical series unspecified as to detail 4 of 10 cases with intestinal anastomoses treated with ACTH developed suture line leakage(9). Ragan has stated that the depression of wound healing by ACTH is a function of the dosage, the larger doses inducing depression where lower doses do not (10). The purpose of this study was to determine the effect of varying doses of ACTH on end to end anastomoses of the colon performed with minimal soiling of the peritoneal cavity at the time of operation.

Method. The experimental animals were healthy male mongrel dogs fed on ordinary kennel rations prior to surgery. Ten test animals and 10 control animals weighing from 6.4 to 18.9 kg were subjected to identical procedures following a 24-hour fast. Under ether-oxygen anesthesia the abdomen was entered through a left paramedian incision. The transverse colon was transected between the right colic and middle colic arteries without disturbing the marginal artery. The clamped cut ends of the colon were closed with Parker-Kerr basting stitches. Using fine silk on straight intestinal needles, the anastomosis was performed with a single row of Halsted stitches approximately 3 mm apart. After removal of the basting stitches the abdomen was closed in 3 layers. Postoperatively all animals were fed milk *ad lib.* immediately and for 7 days before sacrifice. After closure of the abdomen each animal in the test series was given an injection of 160 units of repository ACTH (Parke-Davis x5964) subcutaneously. A similar injection was given daily for 7 days. As the weight of the animals varied, the amount represented dosages of from about 8 to 23 units of ACTH per kilo per day. No antibiotics were used. Samples of blood were drawn at intervals and examined for total protein, hemoglobin, and eosinophiles. The animals were observed daily for signs indicative of peritonitis from suture line leakage. On the 7th postoperative day each animal was killed with ether. After examination of the contents of the abdomen a 10 cm loop of colon containing the anastomosis was removed and tested for bursting strength by the Harvey method. With the ends of the loop closed the intraluminal pressure was increased at a rate of 10 mm of Hg/second by compressed air until the first bubbles of gas were seen escaping from the submerged specimen. The pressure at this instant and the site of the leak were noted.

Results. The total protein and hemoglobin determinations revealed slight drops from preoperative values within a range of 1 g/100 cc in both treated and control animals. The eosinophile counts of the animals given ACTH fell an average of 99% on the first postoperative day and remained at this low level for the duration of the experiment. The eosinophile counts of the control animals showed a variable change from preoperative values, fall-

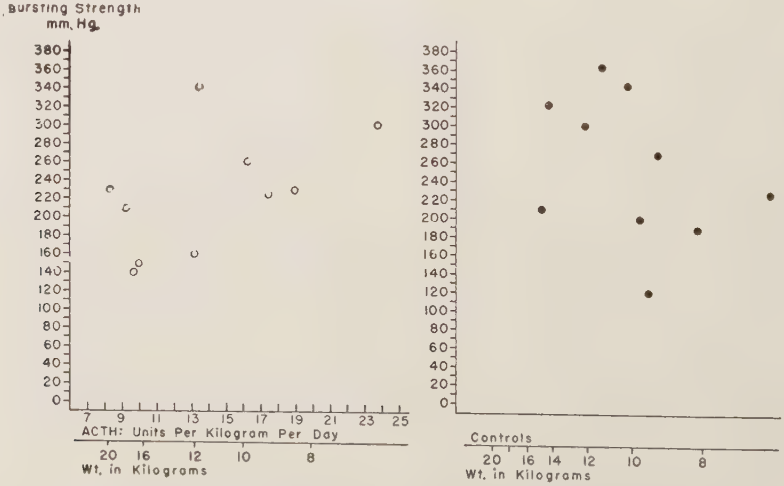

FIG. 1. Seventh day bursting strengths of end to end anastamoses of the colon. Comparison of values from animals given ACTH with those of control series.

ing from 10 to 50%, as has been noted in patients undergoing surgery(11).

No difference was noted in the postoperative course of the animals in either group. Suture line leakage was not induced with doses of ACTH as high as 23.4 units/kg/day. The bursting strength of the anastomoses in the control series ranged from 120 to 365 mm Hg with an average of 257 mm Hg and a mean value of 242 mm Hg. The bursting strength of the anastomoses in the ACTH series ranged from 140 to 340 mm Hg with an average of 224 mm Hg and a mean value of 240 mm Hg. The site of the first leak under pressure was always at the suture line. No correlation was evident between the dosage of ACTH and the anastomotic bursting strength. For example an animal receiving 23.4 units of ACTH/kg had an anastomotic bursting strength of 300 mm Hg, while an animal receiving 10.6 units/kg/day had an anastomotic bursting strength of 150 mm Hg (Fig. 1).

Discussion. The healing curves of intestinal anastomoses tested with and without suture support intersect at the 7th day as shown by Fellows *et al.*(12). It would seem, therefore, that the presence of the sutures in the anastomoses did not alter the experimental results of this study. The bursting strengths found in these experiments correspond to those found in previous studies of anastomoses of the colon(13). Doses of ACTH such as used in this investigation are far in excess of those used clinically, the highest being equivalent to over 1600 units per day in an adult patient.

Summary. Suture line leakage was not observed in a series of 10 dogs given large daily doses of ACTH following end to end anastomosis of the colon. The strength of the suture lines was not significantly altered by ACTH administration as compared with non-treated controls. No correlation was evident between the amount of ACTH given and the strength or gross appearance of the anastomoses.

The ACTH used in this study was supplied through the generosity of the Parke Davis Co.

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Adaptation of Type I Strain of Poliomyelitis Virus to Mice and Cotton Rats. (21061)

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The adaptation to mice and hamsters of Type I poliomyelitis virus, using the intraspinal route, was reported recently by Li and Schaefer(1). They were unable, however, to adapt their strain to cotton rats, and had no success with the intracerebral route. These authors claimed that continuous mouse passage of the strain reduced its intracerebral pathogenicity for the monkey(1).

The present paper describes the adaptation of a Type I strain of poliomyelitis virus to both mice and cotton rats using either intraspinal or intracerebral inoculation. Some virus sublines thus obtained, proved to be singularly apathogenic for monkeys when given by the intracerebral route.

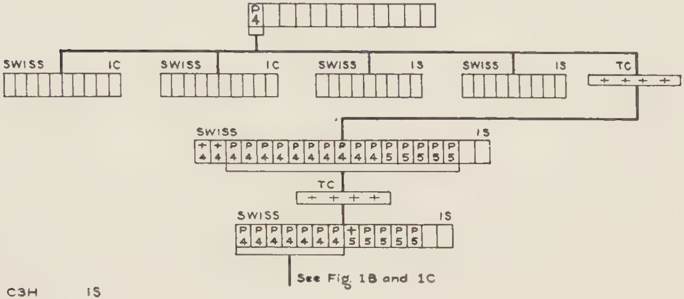
Materials and methods. Virus strains. The Sickle(2) strain was obtained from Dr. Albert Sabin, Children's Hospital Research Foundation, University of Cincinnati College of Medicine, Cincinnati, Ohio, in the form of monkey spinal cord suspension representing the third monkey passage of the virus since its original isolation. The Mahoney(2) strain was procured through the kindness of Dr. Gordon Brown, Dept. of Epidemiology, School of Public Health, University of Michigan, in the form of monkey cord suspension representing their Pool III. Both strains were passaged once in monkey CNS, and the resulting spinal cord suspensions were then used to adapt these strains to tissue cultures. *Tissue cultures.* The technic was similar to that currently used in most laboratories(3,4). Fragments of monkey (Java) kidney tissue, embedded on the walls of roller tubes, were immersed in 199 nutrient medium(5). *Animals.* Swiss mice, cotton rats, and Java and rhesus monkeys were obtained from commercial dealers. The following inbred strains of mice were used: dba, C3H carrying the milk factor (C3H+), C3H free of the milk factor (C3H–), F-1 generations representing crosses between dba and C3H+, PRI and ICR (Swiss strain specially inbred at the Institute for Cancer Research, Fox Chase, Philadelphia, Pa.). All these strains were bred in this laboratory. Original stocks, with the exception of PRI, were obtained from Dr. T. Hauschka of the above Institute. The PRI mice were furnished by Dr. Albert Sabin of the University of Cincinnati. *Pathologic examination* of the central nervous system was performed usually 4-5 weeks after inoculation, using the method of Nissl in celloidin imbedded material. For quantification of cellular losses in the anterior horn of the spinal cord, Bodian's procedure(6) was used.

Experimental. Adaptation of the virus to PRI mice. Undiluted tissue culture fluid infected with the 8th tissue culture passage of Sickle strain was mixed in equal proportions with fluid infected with the 10th tissue culture passage of the Mahoney strain, and the mixture was injected intracerebrally into 12 Swiss mice. A single animal was found paralyzed on the 4th day after inoculation (Fig. 1A). A 10% suspension of its spinal cord was injected intraspinally into mice and inoculated into tissue culture. Although cytopathogenic effect was noted in the latter, no signs of sickness were observed in the injected mice. However, inoculation of tissue culture supernatant into mice intraspinally caused paralysis in 17 out of 19 animals (Fig. 1A). One more alternate passage between tissue cultures and mice resulted in 12 out of 14 animals becoming infected. The spinal cords of 7 paralyzed mice were made into a 10% suspension and inoculated intraspinally into groups of mice representing 7 different genetic strains. When and if inoculated animals became paralyzed, their spinal cords were removed, made into a 10% suspension and injected intraspinally into mice of the homologous strain. The results of the ex-

Adaptation of SM Virus to PR1 Mice

Equal Volumes Mahoney 10th TC Passage Plus Sickle 8th TC Passage

Fig. 1A



C3H IS P = C3H or other mouse strain injected intraspinally and found paralyzed

C3H IC P = C3H or other mouse strain injected intracerebrally and found paralyzed

TC + + + + = 4 plus cytopathogenic effect in monkey kidney culture

TC O = Complete lack of cytopathogenic effect in monkey kidney culture

Blank space inside of figure idicates no signs of illness

PP = Partially paralyzed IS = Intraspinal + = Dead

P = Completely paralyzed IC = Intracerebral

Fig. 1B*

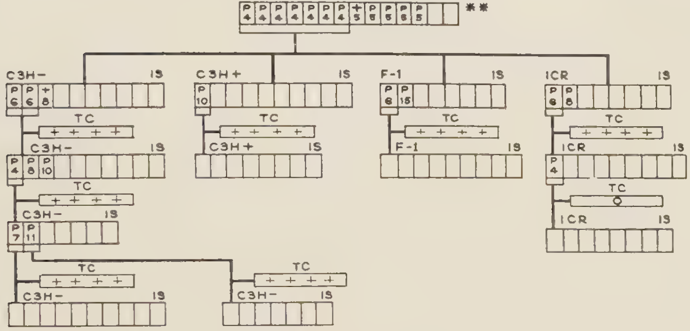
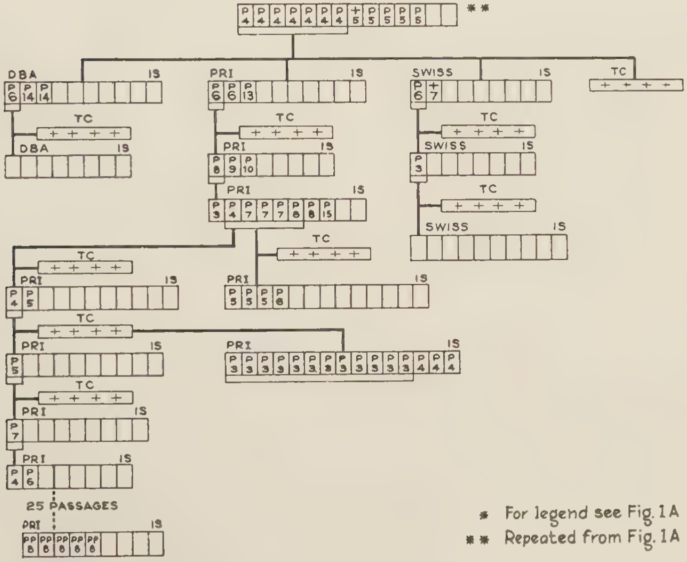


Fig. 1C *

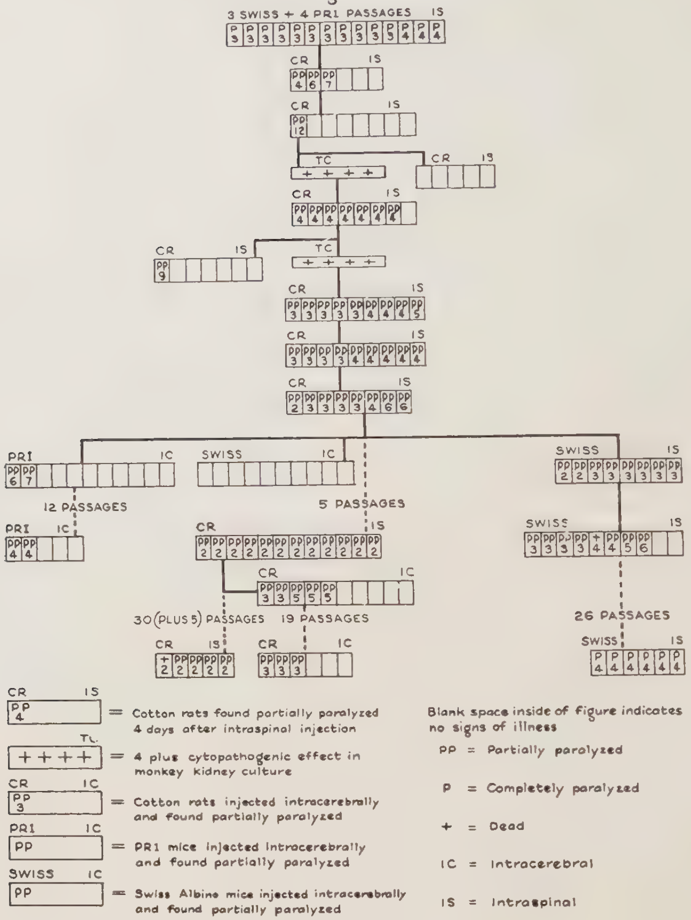


* For legend see Fig. 1A

** Repeated from Fig. 1A

Adaptation of SM Virus to Cotton Rats

Fig. 2


3 SWISS + 4 PRI PASSAGES IS
CR IS
TC
12 PASSAGES
5 PASSAGES
30 (PLUS 5) PASSAGES
19 PASSAGES
26 PASSAGES
PRI IC
SWISS IC
SWISS IS
= Cotton rats found partially paralyzed 4 days after intraspinal injection
= 4 plus cytopathogenic effect in monkey kidney culture
= Cotton rats injected intracerebrally and found partially paralyzed
= PRI mice injected intracerebrally and found partially paralyzed
= Swiss Albino mice injected intracerebrally and found partially paralyzed
Blank space inside of figure indicates no signs of illness
PP = Partially paralyzed
P = Completely paralyzed
+ = Dead
IC = Intracerebral
IS = Intraspinal

periments are shown in Fig. 1B and 1C. It may be seen from the paralysis ratio that the susceptibility of different breeds of mice was about the same at the first passage level. However, beyond the second passage the virus could be propagated in only 2 strains of mice: the C3H– (Fig. 1B) and the PRI (Fig. 1C). The infectivity faded away in the C3H-mice after the third consecutive passage, whereas the propagation of the virus in PRI mice was maintained in an unbroken sequence. Up to date, the virus has been propagated through 25 serial passages in PRI mice injected intraspinally with 10% suspensions of infected cord tissue. At this level the incubation period varies between 5 and 8 days, and usually more than half of the inoculated mice show signs of paralysis. The cytopathic titer of the virus in CNS of the mouse varies from $10^{-4.00}$ to $10^{-5.00}$, and one passage through tissue culture medium increases the pathogenicity of the agent for PRI mice.

Adaptation of virus to cotton rats. Material representing the seventh mouse passage (Fig. 2) was introduced intraspinally into 6 cotton rats. Three of these animals became paralyzed 4 to 7 days after inoculation, and their spinal cord tissue was injected into 8 cotton rats. As shown in Fig. 2, one of these animals sickened on the 12th day after inoculation and its cord tissue was inoculated into tissue culture tubes and intraspinally into five cotton rats. Although as noted in Fig. 2, cytopathic effect was observed in tissue cultures, no signs of sickness were elicited in the inoculated cotton rats. However, the intraspinal inoculation of the tissue culture supernatant into cotton rats caused paralysis in 7 out of 8 animals. After one more alternate passage through tissue culture, the virus became adapted to cotton rats, and the resulting strain was maintained in an unbroken sequence in 3 main sublines.

Cotton rat passage subline. In one series (Fig. 2), the SM virus was propagated through 30 passages in cotton rats injected intraspinally. At this level the incubation period varied from 2 to 3 days, and all the animals inoculated intraspinally with a 10% suspension of spinal cord tissue became paralyzed. In a series not shown in the Figures material representing the 32nd cotton rat passage of the virus was injected *intracerebrally* into a group of cotton rats, and has been propagated in this fashion through 10 consecutive passages up to date. The incubation period in animals injected intracerebrally varies from 3 to 7 days, and usually 60-80% of animals injected with a 10% suspension of infected brain and spinal cord tissue become paralyzed. The other "intracerebral" series originated from the sixth intraspinal passage (Fig. 2). In this subline, the virus has been maintained through 19 consecutive intracerebral passages, using infected brain tissue as virus source. The incubation period is from 3 to 4 days, and almost all of the animals injected with a 10% suspension of brain tissue die from infection. In the first cotton rat series, virus representing the 32nd intraspinal cotton rat passage was adapted to Swiss albino mice injected intracerebrally. So far, the virus has been passaged without difficulty through 6 consecutive mouse passages using cord tissue as inoculum (not shown in the figure). The incubation time is from 3 to 6 days, and the mortality ratio is not uniform, although the series has been maintained in unbroken sequence.

TABLE I. Serological Identity of SM Strain Results of Neutralization Tests in Tissue Culture.

Passage	Dilution	No. of cytopathic units	Antiserum type	Ratio of TC tubes with cytopathic effect†
7 M + 1 CR*	1:10	N.T.	I	0/2
	1:100		I	0/3
	1:10		None	3/3
	1:100		None	3/3
+ 5 CR*	1:20	50000	I	0/3
			II	3/3
			III	3/3
			None	3/3
+ 41 CR*	1:10	5600	I	0/6
			II	6/6
			III	6/6
			None	6/6

M = mouse passage; CR = cotton rat passage.

* Plus one tissue culture passage.

† Denominator = No. of tubes inoculated with serum and virus; numerator = No. of tubes showing cytopathic changes.

Swiss albino mouse passage subline. Cotton rat cord suspension representing the 6th cotton rat passage of SM virus was injected into 8 Swiss mice intraspinally (Fig. 2). All of the mice became paralyzed on the third day after inoculation, and their spinal cord tissue was used as inoculum for another group of Swiss mice (Fig. 2). When 8 out of 10 animals in this group became paralyzed, their cord tissue was used to initiate a series of passages in mice which has been carried up to date through 26 consecutive generations. After intraspinal inoculation with a 10% suspension of mouse spinal cord tissue, mice show signs of paralysis on the third to fourth day. All animals died of infection.

PRI mouse passage subline (intracerebral series). This subline was initiated with starting material from the sixth cotton rat passage (Fig. 2) injected intracerebrally into mice of the PRI strain. As indicated in Fig. 2, this series has now been maintained for 12 consecutive passages. The incubation period varies from 4 to 6 days, but usually less than half of the inoculated mice show signs of paralysis.

Identification of the SM strain. Table I summarizes results of neutralization tests in tissue culture with SM virus representing three different passage levels. Antisera were prepared in this laboratory in rhesus monkeys following the technic described by Salk(7). These sera were found to be type specific when submitted to numerous neutralization tests prior to actual use for identification purposes. It may be observed from the results summarized in Table I that the SM virus retained the serological identity of a Type I strain throughout the series of passages in

TABLE II. Infectivity of Various Sublines of SM Virus.

	No. of passages in—					Infectivity titer† in—		
	Cotton rat		Mouse					
Subline*	i.s.	i.c.	Swiss	PRI	Tissue‡	Cotton rat	Mouse	Tissue culture
Cotton rat	10	16	—	—	B	3.50	2.10	5.50
					C	2.00	1.00	4.50
	32	6	—	—	B	3.00	1.40	4.50
					C	3.40	2.40	5.50
Swiss mouse	6	—	25	—	B	<1.00	<1.00	3.20
					C	2.50	1.50	4.20
PRI mouse	—	—	3	24	B	<1.00	<1.00	5.20
					C	<1.00	1.20	5.50

* For information concerning various sublines see text and also Fig. 1A, 1C and 2.
† Expressed as negative log to the base of 10.
‡ B = brain; C = cord.

mice and cotton rats. Five rhesus monkeys were inoculated intracerebrally with SM virus representing the third cotton rat passage (Fig. 2). Two of the animals were sacrificed while paralyzed. The other 3 were challenged 30 days later with 100 PD_{50} of Type I virus (Brunhilde strain, monkey cord tissue). None of these monkeys showed signs of paralysis, whereas 8 out of 9 non-immunized control monkeys became paralyzed.

Comparative infectivity of different sublines of SM virus for mice and cotton rats. As seen in Table II the infectivity of the 2 cotton rat sublines and that of the PRI mouse passage are similar when measured by the cytopathic effect in tissue culture, while the infectivity of the Swiss mouse passage line is lower. The virus content of brain was lower than that of spinal cord in all sublines except the one representing the cotton rat series with the highest—19—number of intracerebral passages. Virus maintained solely by serial passage through mice of the PRI line is apathogenic for cotton rats, of low virulence for mice, and of high infectivity for tissue culture. Virus maintained by serial passage through Swiss mice after 6 passages through cotton rats retained its infectivity for cotton rats (spinal cord tissue). Brain tissue material representing two virus lines which are maintained through intraspinal inoculation of mice is non-pathogenic for either cotton rats or mice, although the fact that it contains virus is indicated by cytopathic effect in tissue culture. Conversely, the virus content of brain tissue of cotton rats injected intracerebrally can be determined by titration in either mice or cotton rats. The virus titer, however, apparently increases with the number of intracerebral transfers.

Comparative pathogenicity of the various sublines for monkeys. As seen in Table III, the SM subline propagated in cotton rats was as virulent for monkeys as the original unadapted strain, as judged from the clinical manifestations and the pathologic findings (Table IV). The cellular loss was apparently irrespective of the virus dilution. The *Swiss mouse subline* showed little virulence for monkeys (Table III). At the 12th passage level none of the 5 monkeys inoculated showed clinical manifestations. Pathologic examination disclosed no appreciable cellular loss and no evidence of inflammatory changes. However, at the 22nd passage level 2 monkeys out of 15 injected with different dilutions of the virus became paralyzed; loss of cells in the anterior horn was 62 and 20% respectively. The other 13 monkeys showed no pathologic evidence of poliomyelitis. A moderate degree of paralysis was also observed in 2 rhesus monkeys representing a group of 12 animals inoculated with dilutions of tissue culture supernatants infected with the 35th Swiss mouse passage of the virus. The other 10 monkeys showed neither clinical nor pathologic evidence of poliomyelitis. The *PRI mouse subline*, as seen in Table III, shows no virulence for either rhesus or cynomolgus monkeys. In 3 separate experiments, material representing different passage levels of the virus failed to elicit signs of illness in any

TABLE III. Virulence of SM Rodent-Adapted Strain of Poliomyelitis (Type I) for Monkeys.

Inoculum					Paralytic ratio of monkeys injected with dilutions of virus								
		PD$_{50}$ titer§ in—											
Host*	Passage No.	Mice	Cotton rats	TD$_{50}$‖ titer	10^{-0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	Monkey species	Route of inoculation
Cotton rat	12 + 11 TC	2.75	N.T.	6.70	2/4	4/4	4/4	3/4	1/4	0/4	0/4	Java	Intracer.
	16	2.80	N.T.	5.50	—	8/10	1/2	2/2	1/2	2/2	—	Rhesus	
Mouse,† Swiss strain	12	2.75	N.T.	5.50	—	0/5	—	—	—	—	—	"	
	22‡	1.00¶	2.15	4.50	—	2/4	0/3	0/2	0/2	0/2	0/2	"	
	35 + 1 TC	N.T.	N.T.	6.00	1/3	1/3	0/3	0/3	—	—	—	"	
Mouse, PRI strain	26 + 8 TC + 1 mouse	1.50		5.20	—	0/4	0/4	—	—	—	—	"	"
	22 + 1 TC‡ + 1 mouse	2.00	<1.00	5.20	—	0/4	0/4	0/4	0/4	0/2	—	Java	"
	28 + 1 TC + 1 mouse	1.20	N.T.	3.20	—	0/4	0/4	—	—	—	—	"	"
					—	0/4	0/4	—	—	—	—	"	Intrav.
					—	0/4	0/4	0/4	0/4	0/4	—	Rhesus	Intracer.
					—	0/4	—	—	—	—	—	"	Intrav.

* Virus previously passaged through 3 Swiss Albino and 4 PRI mice. † Six passages through cotton rats preceded the mouse passage line.
‡ Used for oral administration to human subjects. § Expressed as negative log to base of 10. ‖ Cytopathogenic effect in tissue culture.
¶ Mice found paralyzed at 10^{-1} dilution.
TC = Tissue culture passage. N.T. = Not tested.

TABLE IV. Histopathologic Changes (Cell Loss) Observed in Spinal Cord Tissue of Cynomolgus Monkeys Injected I.C. with Dilutions of SM Strain.*

		Cell loss, %	
Monkey	Virus dilution	Cervical	Lumbar
30–33	10^{-1}	49	46
30–34	"	78	82
30–36	"	35	37
30–38	"	74	58
30–39	"	37	70
30–43	10^{-2}	60	35
30–45	10^{-3}	46	44
30–46	"	80	41
30–47	10^{-4}	60	65
30–49	10^{-5}	29	26

* Representing 16th cotton rat passage (see Table III).

of the 54 intracerebrally injected monkeys. Histologically, in none of these animals was there found appreciable loss of neurons or inflammatory reaction. The avirulence of the virus was further confirmed in the last experiment (Table III) when groups of cynomolgus or rhesus monkeys were injected intravenously with infected mouse cord suspension. This route of inoculation was chosen since it made possible the administration of a large quantity (5 ml of 10% suspension of mouse spinal cord) to an animal, and because monkeys had previously been observed to be especially susceptible to that particular route of infection. As seen in Table III, none of the intravenously injected monkeys showed signs of poliomyelitis, and the results of histopathologic examinations were equally negative.

The attenuation of the SM virus may have occurred as a direct result of selection of avirulent (for monkeys) mutants in the course of propagation in the central nervous system of the PRI mouse. An alternative hypothesis is that while the genetic units of the SM strain remained undisturbed, characteristics of the virus population changed under the direct influence of the cellular ambient (PRI mouse CNS) in which the virus propagated. If the latter hypothesis concerning non-genetic changes be correct, then passage of the PRI subline in another host cell would induce changes in its pathogenic properties. This did not occur, however, when virus representing the 26th PRI mouse passage (Table III) was passaged through eight consecutive passages in cynomolgus renal epithelium cells grown in tissue culture. It would seem, therefore, that the attenuation of SM virus might well be considered as directly attributable to the outgrowth, in the course of passages through PRI mice, of selective mutants avirulent for monkeys.

Summary. A mixture of 2 strains of Type I poliomyelitis virus was adapted to the PRI strain of mice and to cotton rats. The virus could be propagated in certain sublines when either the intraspinal or intracerebral route of inoculation was used. The infectivity of different sublines for mice and cotton rats varied, but the cytopathic titer in tissue culture was of similar magnitude. The passages in PRI mice resulted in a subline completely avirulent for rhesus and cynomolgus monkeys.

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Administration of an Attenuated Type I Poliomyelitis Virus to Human Subjects. (21062)

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The preceding paper(1) describes the adaptation of a Type I strain of poliomyelitis virus to mice and cotton rats, and presents evidence of attenuation of the virus for monkeys injected intracerebrally. It has been previously reported that a Type II rodent adapted strain of similarly attenuated properties, when administered orally to 85 non-immune human subjects, produces persistent immune response(2-4). This report describes the results of feeding Type I mouse adapted strain to 2 human subjects, and a mixture of Type I and Type II strains to one subject. These 3 individuals were affected with fatal neoplastic disease, and none had antibodies against the viruses fed.

Materials and methods. Virus. Two pools of infectious material were used for individuals who received only Type I virus: one represented the 22nd passage of the Swiss mouse subline of the combined Sickle and Mahoney strains(1), and the other represented the 22nd + one tissue culture + one mouse passage of the PRI mouse passage line. As stated elsewhere(1), both preparations had been found to have little or no pathogenicity for monkeys injected intracerebrally. Each feeding dose consisted of one ml of a 20% suspension of infected mouse spinal cord tissue diluted in one ounce of milk. The third individual received 4 ml of a virus suspension consisting of equal parts of the SM strain (Type I) and the TN strain (Type II). The SM strain represented the 28th PRI mouse + TC + one mouse brain passage of the virus(1), and the TN strain the 8th mouse brain passage of the virus(2).

Clinical observations, collections of blood and stool specimens. These were conducted according to methods described in detail before(2,3).

Method of virus assay. For determination of the presence of virus in stools or blood, 6 tissue culture (cynomolgus monkey renal epithelium) tubes were used for each specimen according to the method described by Horstmann(5). If all 6 tubes showed no cytopathic effect, 2 blind passages were made before a specimen was considered negative. For titration of virus suspensions, 3 tubes were used for each dilution. The general technic was the same as described in the preceding paper(1).

Neutralization tests. Aliquots of serial 4-fold dilutions of test serum were mixed in equal amounts with dilutions of virus containing 20-60 TD_{50} of Mahoney strain (Type I) and, in the case of combined feeding, an equal dosage of TN (Type II) strain. Also in the instance of dual feeding, the stool isolate was not only tested against each specific serum, but also against a mixture of antisera of the 2 types. After incubation at room temperature for one hour, the suspensions were inoculated into tissue culture tubes which were observed for cytopathic effect. If each antiserum tested separately failed to neutralize the cytopathic effect of the virus, but the mixture of the 2 sera did prevent cytopathic effect, the conclusion was reached that 2 types of virus were present simultaneously.

Results. None of the 3 individuals showed any apparent signs or symptoms of illness which could be attributed to the ingestion of the virus. No virus was isolated from blood specimens of the 3 subjects collected daily during the 8 days following ingestion of the virus (Table I). Intestinal carriage was established in all 3 individuals, as indicated by the isolation of Type I virus from stool samples of the 2 subjects who received the SM strain alone, and by the isolation of Type I and Type II virus from the stools of the person who received the viral mixture.

Homologous neutralizing antibodies were observed in the serum of individual No. 1 as early as the 8th day after ingestion of the virus, and a rise was noted in blood collected 14 days after feeding of the virus. The serum of subject No. 2 had no demonstrable antibodies on the 8th day after administration of the virus, but antibodies appeared in the blood between the 14th and 21st day. The serum of subject No. 3 obtained on the 8th day after feeding contained antibodies against Type I, but not against Type II. Seven days later, the serum of this subject was found to neutralize both Type I and Type II viruses and

TABLE I. Occurrence of Intestinal Carriage, Absence of Viremia, and Serological Evidence of Immunity in 3 Human Subjects after Oral Administration of Type I (SM strain) and Type II (TN strain) Poliomyelitis Virus.

Human subject No.	Inoculum		Stools collected and processed	Virus isolated from stools	Viremia		Antibody response					
								50% serum titer				
								Days after feeding				
	Virus	TD_{50} titer			Blood tested	Virus isolated from blood	Virus type	0	8	15	20	33
			Days after feeding									
1	SM*	4.50	4, 7, 10, 15, 25, 36	4, 7, 10, 15, 25	4, 5, 6, 7, 8	None	I	<1:2	>1:2	1:32	—	1:22
2	SM†	5.20	5, 7, 13, 28, 46, 70	5, 7, 13, 28, 46	1, 2, 3, 4, 5, 6, 7, 8	”	I	<1:2	<1:2	1:2	1:32	1:32
3	SM +	3.20	§3, 11, 17, 37	3,‖ 11,‖ 17§	1, 2, 3, 4, 5, 6, 7, 8	”	I	<1:2	1:8	1:22	1:11	1:365¶
	TN‡	1.50					II	<1:2	<1:2	1:8	1:64	1:64

* Received 22nd Swiss mouse passage of the virus. } See preceding paper(1).
† ” ” PRI + 1 TC + 1 mouse passage of the virus. } See preceding paper(1).

‡ Received 4 ml of suspension containing equal parts of: (1) SM (Type I) virus, representing 28 PRI + 1 TC + 1 mouse passage of the strain and (2) TN (Type II) virus, representing 8th mouse brain passage of the strain.

§ Type II isolated only.

‖ Both Type I and Type II viruses isolated from the stool suspension.

¶ Observed on the 63rd day.

the antibody titer against Type I became higher on the 63rd day.

Stool specimens collected from subject No. 1 on the 7th and 10th days after administration of the virus were pooled and made into a 10% suspension according to a process described previously(2). The same was done with stool specimens collected from subject No. 2 on the 7th and 13th days after ingestion of the virus, except that the virus content of the stool suspension was concentrated 10-fold by means of differential ultracentrifugation (6). Aliquots of 10-fold dilutions of the 2 stool suspensions (one in concentrated form) were then injected intracerebrally into rhesus monkeys. Table II shows the paralytic ratio of monkeys thus injected, as well as results of titration of the viral pools used in the original feeding of the individual(1). The results indicate that the SM virus, Swiss mouse passage line, after one passage through the human intestinal tract, failed to acquire pathogenic properties for rhesus monkeys injected intracerebrally. In these 18 animals there was no pathologic evidence of poliomyelitis. Fecal material obtained from individual No. 2 (PRI mouse passage line), concentrated 10 times, produced mild poliomyelitis in 2 monkeys which were injected intracerebrally with the undiluted concentrate. However, there was no clinical or pathologic evidence of involvement of the central nervous system in 16 monkeys which were injected intracerebrally with serial 10-fold dilution of the concentrate. Although the TD_{50} titer of the stool suspensions collected from the 2 subjects was lower than that of the original inoculum, this fact alone cannot account for the lack or low intracerebral pathogenicity of the material for rhesus monkeys, since in a study of another strain of poliomyelitis virus infected human stool suspensions with a TD_{50} titer of $10^{-1.50}$ were observed to be pathogenic for rhesus monkeys injected intracerebrally.

Summary. Two human subjects who had no antibodies against Type I poliomyelitis virus were fed a mouse adapted Type I strain, and one human subject who had no antibodies against Types I and II was fed a mixture of mouse adapted Type I and Type II strains. None of the individuals showed clinical signs

TABLE II. Comparative Pathogenicity of SM Strain of Poliomyelitis Virus for Monkeys before and after Passage through Human Intestinal Tract.

Virus origin				TD_{50} titer	PD_{50} titer in mice	Paralytic ratio of monkeys injected IC with dilutions of virus						
Subline*	Passage	Human sub.	Material			10x†	1.0	2.0	3.0	4.0	5.0	6.0
Swiss mouse	6 CR + 22 Swiss mouse	—	Mouse cord	4.5	1.00	—	2/4	0/3	0/2	0/2	0/2	0/2
	6 CR + 22 Swiss mouse + 1 human	No. 1	Human stool	2.5	<1.00	—	0/4	0/4	0/4	0/4	0/4	—
PRI mouse	22 PRI + 1 TC + 1 mouse	—	Mouse cord	5.2	2.00	—	0/4	0/4	0/4	0/4	0/2	—
	22 PRI + 1 TC + 1 human	No. 2	Human stool concentrate	3.8	<1.00	2/2	0/4	0/4	0/4	0/4	0/4	—

* See preceding paper(1).

† 10x concentrated by differential ultracentrifugation.

CR = Cotton rat passage. TC = Tissue culture passage.

of illness as a result of ingestion of the virus. In the absence of viremia, all 3 excreted virus in the stools. Homologous neutralizing antibodies developed in the sera of each person. Virus passaged through the human intestinal tract maintained the very low pathogenicity for monkeys when injected intracerebrally.

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Chronic Toxicity Studies on Conidendrins and Conidendrols. (21063)

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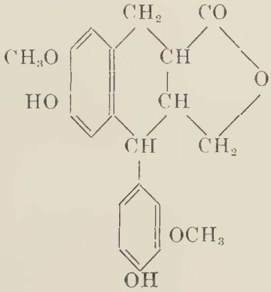
The importance of efficient, non-toxic antioxidants in foods subject to oxidative deterioration is unquestioned. Recently, it has been suggested(1-3) that α- and β-conidendrols could be used as effective antioxidants in edible fats, provided they cause no toxic reactions. This paper reports toxicity studies on these conidendrols, and on α- and β-conidendrin. α-Conidendrin is a partially methylated polyphenol occurring in a number of coniferous trees. (For a description of its occurrence, see Brauns(4)). It can be obtained from the sulfite liquor of the wood-pulp industry. Its chemical structure is:



By inversion at the carbon adjacent to the carbonyl group of the lactone ring, an isomer, β-conidendrin, is formed. Both of these compounds are mild antioxidants. α- and β-Conidendrol (also called α- and β-nonconidendrin) likewise are isomers, and are obtained by demethylation of α-conidendrin(5). They are stronger antioxidants than the conidendrins. Both conidendrols inhibit the rate of peroxide formation in fat when incorporated in the fat in concentrations of 0.01 to 0.1%. The β-isomer is the more effective as a fat antioxidant(3).

Reports on toxicity of these compounds seem to be limited to a short statement(6) on subacute toxicity following intraperitoneal administration to mice. Such administration for several days showed the maximum tolerated daily dose of α- and β-conidendrin to be 500 and 250 mg/kg, respectively, and of α- and β-conidendrol, 62.5 and 113.5 mg/kg, respectively. No statement has been found concerning the metabolism of these compounds in the mammal. However, there are reports that degradation of α-conidendrin can be accomplished by microorganisms(7), although identification of the metabolites has not yet been announced, and that a polyphenol oxidase of microbial origin can degrade conidendrin(8).

Experimental. The effect of continued feeding of diets containing these 4 compounds was studied on weanling albino rats.* The

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* The conidendrins and conidendrols were kindly supplied by Drs. W. W. Moyer and W. M. Hearon, Crown Zellerbach Corp., Camas, Washington.

TABLE I. Body Weights of Rats on Diets Containing Conidendrin or Conidendrol.

Compound	Conc. in diet, %	No. of rats	Mean body wt ± S.E.	Exp. minus control ± S.E.	"t"	"P"
			Males			
Control diet	0	9	346 ± 16.8			
α-CNN	.5	9	344 ± 8.0	−2 ± 18.6		
"	1.0	9	359 ± 5.3	13 ± 17.7		
β-CNN	.5	6	327 ± 16.4	−19 ± 23.5		
"	1.0	8	312 ± 12.8	−34 ± 21.1	1.61	
α-CNL	.5	9	353 ± 12.5	7 ± 20.9		
"	1.0	10	349 ± 4.1	3 ± 17.3		
β-CNL	.5	8	353 ± 7.2	7 ± 18.3		
"	1.0	10	307 ± 6.0	−39 ± 17.9	2.18	.05
			Females			
Control diet	0	10	233 ± 6.1			
α-CNN	.125	4	281 ± 14.6	48 ± 15.9	3.02	.01
"	1.0	5	265 ± 9.2	32 ± 11.0	2.91	<.02
β-CNN	.125	5	260 ± 8.1	27 ± 10.2	2.65	.02
"	1.0	5	251 ± 8.9	18 ± 10.8	1.67	
α-CNL	.125	5	232 ± 8.3	−1 ± 10.4		
"	1.0	5	233 ± 9.5	0 ± 11.3		
β-CNL	.125	5	238 ± 9.0	5 ± 10.9		
"	1.0	5	243 ± 4.2	10 ± 7.4	1.35	

S.E. = Stand. error. "t" = Student's t, not recorded unless it is greater than 1. "P" = probability, not recorded unless it is 0.05 or less. CNN refers to conidendrin, CNL to conidendrol. Data for males is for 190 days after the start of the experiment; for females, 200 days.

basic diet consisted of, in percent: corn meal, 73; linseed oil cake meal, 10; alfalfa meal, 2; casein, 10; cod liver oil, 3; bone ash, 1.5; and NaCl, 0.5. The substances under study were added to this basic diet with thorough mixing. Water and food were available *ad libitum.* Concentrations of the substances under study were, for the females, 0.125 and 1.0% of the diet, and for the males, 0.5 and 1.0%. Preliminary experiments suggested that studies of lower concentrations were not necessary. Initially, there were 5 females in each group (except for control group which contained 10 rats), and 10 males per group, 5 animals to each cage. Weekly determinations of body weight, food intake and general appearance were made for 200 days through the rapid growth phase of the animals. Appearance was normal for animals on all of the compounds at all concentrations. Body weights towards the end of the 200-day period are recorded in Table I. (For convenience in calculating, the weights of the males were taken after 190 days on the experimental regimen, while for the females, the time was 200 days.) Statistically, it would appear that one group of males was significantly lighter in weight than the controls, and that 3 groups of females were significantly heavier than their controls. In view of the small number of females in each group, the lack of correlation between dosage levels, and the lack of correlation between sexes, it seems doubtful that the differences are real. Two hundred and twenty days after the start of the feeding, half of the males were autopsied. There was no gross appearance of tissue damage. Organ weights of these animals did not differ significantly from those of control animals of equal body weight, with the possible exception of the livers and kidneys of the rats receiving 1% of α-conidendrol in the diet. For these 2 organs, the livers averaged 16.5 ± 6.3% heavier than controls, and the kidneys, 11.5 ± 5.1% heavier, with P values of about .05.

Results. The remaining animals were continued on their respective diets for an additional 200 days. They appeared normal throughout this period. Hemoglobin values at the end of this 420-day feeding period were within the normal range for the males; hemoglobin values were not determined for the females. Tissues at autopsy appeared grossly normal except for stones in the kidney pelvis of 2 males, one on each dosage level, receiving α-conidendrol, and several cystic kidneys and yellowish livers in males receiving 1% of β-conidendrol. Tissues from the 420-day animals were prepared for microscopic examination, using a hemotoxylin and eosin stain. The following tissues were examined: heart, lung, liver, spleen, kidney, adrenal, bladder, pancreas, ovary, testis, thyroid, ileum, stomach and, frequently, parathyroid.

In tissues from rats which had received the conidendrins, no changes were found which could be regarded as a direct result of the conidendrin administration. A number of the animals had pneumonia, occasionally with lung abscesses or bronchiectasis, but these animals were in the minority at any dose level. Occasional other changes, such as testicular atrophy, focal pancreatitis, collections of lymphocytes in the kidney and in the heart bore no recognizable relationship to the treatment and were apparently incidental changes.

In the livers of the male rats receiving the higher concentration of β-conidendrol, there was fine vacuolation of the liver cells. In places there were also larger vacuoles. In one animal the change was severe, and there were also small groups of mononuclear cells in the stroma, with minimal increase in fibrous tissue. The change occurred predominantly at the periphery of the lobules. No necrotic cells were identified. Although not specifically tested, the material in these vacuoles was probably fat; this would account for the yellowness of these livers noted grossly. Evidence that severe injury was not associated with this change in liver cells was seen in the form of several mitotic figures in cells containing moderate numbers of fine vacuoles.

A mild similar change was seen in the livers of the female rats which received the larger dose of β-conidendrol. None was seen in the animals on the smaller dose, and only very slight vacuolation of liver cells was seen in 4 of the 10 male animals which received α-conidendrol.

In 3 of the males which received 1% α-conidendrol in the diet. and in 2 on the larger dose of β-conidendrol, there were several small scars in the kidneys, suggesting mild old pyelonephritis. Several of these animals had focal dilatation of renal tubules which contained hyaline casts. One animal which was noted grossly to have a large kidney stone, showed pronounced hydronephrosis. Most of the animals of the male group on these larger doses showed a few more lymphoid cells about the renal pelvis than were present in control animals, but this change was not striking. The kidneys from the females were not distinctly different from controls.

In the females there were no significant changes in the pancreas. In several males on both α- and β-conidendrol there was focal atrophy of acini in the pancreas, sometimes associated with mild local lymphocytic infiltration. These changes could have been results of local duct obstruction, but the observed duct dilatation was not distinctly more than in control animals. A little lymphocytic infiltration of the peripancreatic tissue was sometimes observed. These changes were irregular, without close relationship to the dose of conidendrol.

The other organs showed occasional sporadic changes of various sorts, but nothing which could be attributed to the specific treatment.

The possibility of skin irritation was tested on 4 albino rabbits. Ten per cent ointments of the 4 compounds were prepared by dissolving or suspending the materials in melted Carbowax 1500 W, and stirring while cooling so as to assure uniform distribution in the solidified wax. To each animal approximately 0.1 g of each ointment was applied with gentle rubbing to an area 2 cm in diameter on the clipped skin of the back Except for Saturday and Sunday, applications were made daily for 2 weeks. There was no skin inflammation or other evidence of irritation. At the same time, these ointments were applied to the eye to test for possible irritation of the conjunctiva. Each rabbit received approximately 0.1 g of Carbowax 1500 W in the left conjunctival sac, and an equal amount of one of the ointments in the other eye. These applications were made daily at the same time the skin was being anointed. No conjunctival inflammation was noted in any of the animals. However, a protrusion of the eyeball developed in the right eye of the rabbit receiving α-conidendrin. This was presumably due to formation of an abscess behind the eyeball. Later, the 3 other animals were treated with the α-conidendrin ointment for 2 weeks, with no adverse reactions being noted. It is doubtful if the protrusion of the eyeball in this rabbit was caused by the ointment.

The possibility of skin sensitization by these compounds was studied in albino guinea pigs by the method of Draize, Woodard, and Calvery(9). Test injections did not indicate sensitization, nor were there any cross reactions when an animal prepared with one of the compounds was tested with the other 3 materials.

The mean maximum human intake of fat, according to *Family Food Consumption and Dietary Levels,* U.S.D.A. Miscellaneous Pub. No. 452, p. 194, 1941, is 2.32 lb/person/week, or somewhat less than 0.4 lb/person/day. If all of the dietary fat were treated with conidendrol at the 0.1% level (which, of course, is not possible since much of the fat is in natural dietary products), the human diet would contain 1/150th as much conidendrol per kg of body weight as was ingested by the rats receiving continuously the 1% diet for half of their normal life-span.

Summary. 1. Weanling rats were fed diets containing one of the following antioxidants: α-conidendrin, β-conidendrin, α-conidendrol, and β-conidendrol. The concentrations were, for the females, 0.125 and 1.0% of the diet; and for the males, 0.5 and 1.0% Body weight increase was normal, or approximately normal, during the first 220 days of the experiment, after which frequent weighings were discontinued. 2. Histological examination of the tissues after 420 days on the diets indicated essentially normal tissues, except for the following: In males receiving the higher concentration of β-conidendrol there was vacuolation (presumably fat) of the liver cells, without indication of severe injury. In several of the males receiving the higher concentration of the conidendrols, there were renal changes suggesting mild, old pyelonephritis. In males, several of the rats receiving the conidendrols had focal abnormalities in the pancreas. 3. There was no evidence of irritation to the skin or to the eye of rabbits, and skin sensitization was not developed in guinea pigs. It is concluded that there is at least a 100-fold margin of safety between the amount of conidendrol suggested for fat antioxidant and the amount ingested by rats eating the 1.0% level.

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Passive Transfer of Delayed Hypersensitivity to 2,4-Dinitrochlorobenzene in Guinea Pigs with Leucocytic Extracts.* (21064)

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(Introduced by J. R. Porter.)
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The passive transfer of delayed type skin sensitivity to simple chemical substances(1) and to tuberculin(2-4) has been successful only when mediated by viable leucocytes. Cells that are frozen or heated lose not only their viability but also their ability to transfer passive sensitization. The possibility exists, however, that these methods alter the chemical composition of cellular components as well as the morphological integrity of the cells and that a gentler physical method of treatment might spare the substance responsible for transfer. Sonic vibration would seem to be such a method of cellular disruption, since it has been used effectively by microbiologists and other workers in the preparation of enzymatic extracts from bacteria with a minimum of untoward consequences of various other physical methods. This method should yield preparations showing minimal denaturation, requiring a brief preparative interval, and having less likelihood of thermal alteration of the substance or substances responsible for passive transfer.

This paper summarizes experiments showing passive transfer of delayed type sensitivity to 2,4-dinitrochlorobenzene with extracts of exudative leucocytes disrupted by sonic vibration.

Methods. Guinea pigs weighing 350-500 g were sensitized to 2,4-dinitrochlorobenzene by painting a 2% solution of the chemical in absolute ethyl alcohol on the skin each day for 7 days. Details of sensitization have been described previously(5). Following a latent period of a week a peritoneal exudate was evoked by the injection of 30 ml of light mineral oil. Two days later the exudative leucocytes were collected in heparinized Locke's solution containing 0.2% gelatin and sedimented by centrifugation at 2000 RPM for 15 minutes. Cells from 8-15 donor animals were resuspended in 20 ml of gelatin-Locke's solution. Total leucocyte counts and differential counts on supravital stains were done after the cell suspension was divided into 2 equal portions. Total counts are noted in each experiment. The differential counts were consistent, showing approximately 60% monocytes, 25% lymphocytes, 14% neutrophils, and 1% eosinophils. Half the cell suspension was injected into a control recipient animal, and the other half was subjected to sonic vibration in a 9 KC Raytheon Magnetostriction Oscillator for 5-15 minutes. The temperature of the water jacket was maintained at 30°C ±2. Following this treatment the cellular extract was either injected into a recipient animal or centrifuged at 5,000 RPM for 10 minutes. Centrifugation gave rise to 3 layers: 1) sediment; 2) aqueous supernatant fluid; and 3) surface lipid. The aqueous supernatant layer was injected into a second recipient animal and the combined resuspended sediment and lipid portions into a third. All injections were made by the intraabdominal route. Total leucocyte counts and Giemsa-stained smears were made on both supernatant fluid and sediment to ascertain the degree of cellular disruption. Recipient animals were tested 48 hours after injection by painting with a 1% solution of 2,4-dinitrochlorobenzene in olive oil on the back. The hair on the test area was clipped and the area cleaned with depilatory cream prior to testing. Reactions were observed at 24 and 48 hours following testing and recorded as: 0, no reaction; +, slight erythema; 2+, patchy erythema; 3+, homogeneous erythema; and 4+, marked homogeneous erythema.

Results. The first experiments were de-

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† Public Health Service Research Fellow of the National Microbiological Institute.

TABLE I. Passive Transfer after Injection of the Whole Product of Sonic Vibration in a Single Recipient Animal.

No. of donors	Time of vibration (min.)	WBC/aliquot*	Reactions			
			24 hr		48 hr	
			Vib.	Cells	Vib.	Cells
		×1000				
8	10	1197900	+++	++	+++	++
10	10	1684375	+++	+	+++	++
10	15	1610000	+	+	+	+
15	7.5	1726300	+++	+++	++++	++++
16	6.5	2096300	+	+	++	+++

* Equal numbers of exudative leucocytes were treated with sonic vibration or used intact for control.

0 = no reaction. + = slight erythema. ++ = patchy erythema. +++ = homogeneous erythema. ++++ = marked homogeneous erythema.

Vib. = Animals receiving vibrated cells. Cells = Animals receiving intact cells.

signed to show whether the capability of passive sensitization was retained by cells after treatment with sonic vibration. Leucocytes were collected from 8-16 donors. Half the cells were treated, the other half injected as whole cells for control. The treated cells were injected into a single recipient animal in each experiment. Table I shows that vibrated material is at least as effective as whole cells in the transfer of activity. Smears and total counts of these preparations showed that the method effects complete disruption of leucocytes.

Although no residual intact cells could be seen microscopically, the question of possible transfer by cells still intact after treatment could be obviated by centrifugation of the sonic lysate. In addition the source of transfer activity might be detected by injecting the products of centrifugation separately. In the next group of experiments one recipient animal received the sediment plus the lipoidal fraction, while a second received the aqueous supernatant fluid alone. A third animal served as a control on the intact cells. Table II shows that passive transfer was accomplished in 7 experiments with intact cells and the aqueous supernatant fluid after centrifugation. In only one instance did the sediment possess activity, and this was minimal. Smears and total counts on both the aqueous supernatant fluid and sediment demonstrated that 7.5 minutes was effective in completely disrupting the cells.

Discussion. Owing to the variable nature of passive cellular transfer of hypersensitivity to simple chemical compounds, a control on intact cells from sensitive animals was included in each experiment. In several experiments neither the untreated nor the vibrated cells accomplished transfer. We are at a loss to explain such negative experiments except on the basis of either inadequate donor sensitivity which was not detectable or the heterogeneity of sources of animals used. Such completely negative experiments tend to rule out the possibility that the observed reactions are the result of irritative qualities of the chemical itself. It is significant to note, however, that in cases where sensitivity was transferable with intact cells, it was likewise transferable with extracts.

These results do not preclude the possibility that whole cells involved in transfer may be actively engaged in the production of a sensitizing factor after transfer to a recipient. On the contrary, such productive activity could be the explanation for the lesser intensity of some of the reactions seen in animals receiving fractions of cellular extracts than in those receiving intact cells. However, the exudative cells doubtless have accumulated sufficient amounts of the transfer factor to allow successful passive sensitization.

It is not possible to characterize the factor responsible for transfer at this time. The supernatant fluid after centrifugation at 5,000 RPM for 10 minutes contains particulate matter in addition to soluble constituents. Centrifugation under these conditions could be expected to sediment particles the size of many mitochondria or most bacteria. There-

TABLE II. Passive Transfer after Injection of Separated Portions of Sonic Vibrated Leucocytes into Recipient Animals.

No. of donors	Time of vibration (min.)	WBC/aliquot*	Reactions 24 hr Sup.	24 hr Sed.	24 hr Cells	48 hr Sup.	48 hr Sed.	48 hr Cells
		×1000						
15	6.5	1653000	++	+	++++	++	+	++++
15	7.5	1440827	+	0	+	+	0	++
14	7.5	1285920	+	0	++	++	0	+++
13	7.5	1151702	++	0	++	+++	0	++++
10	7.5	875448	+	0	+++	+	0	+++
10	7.5	625000	+	0	+	+	0	+
10	10	1341316	+	0	+	+	0	+

* Equal numbers of exudative leucocytes were treated with sonic vibration or injected intact for control.

0 = no reaction. + = slight erythema. ++ = patchy erythema. +++ = homogeneous erythema. ++++ = marked homogeneous erythema.

Sup. = Animals receiving aqueous supernatant fluid. Sed. = Animals receiving sediment + lipid fractions. Cells = Animals receiving intact cells.

fore, any particles remaining would be under a size of 0.5 μ.

Attempts to extend this study to other delayed systems have been unsuccessful. In several experiments using tuberculin sensitive donors results were questionable. This may be due to a fundamental difference in the mechanisms of simple chemical and tuberculin sensitivity. However, in none of the tuberculin experiments was an exquisite donor sensitivity achieved, and the degree of sensitivity transferred in control animals was minimal. In experiments in progress we hope to achieve a higher quality of donor sensitivity to enable us to determine whether transfer of tuberculin sensitivity can be accomplished with cellular extracts.

Summary. Passive transfer of delayed cutaneous hypersensitivity to 2,4-dinitrochlorobenzene in guinea pigs has been accomplished with extracts of peritoneal exudative leucocytes disrupted by sonic vibration. Crude extracts of cells treated and subsequently centrifuged were effective as transfer media. Sedimented material was essentially ineffective.

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Hematological Changes Influenced by Short and Long Exposure to Cold. (21065)

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Because of the present interest in studies involving stress and resultant adaptive changes we wish to report our findings with short and long exposures of mice to cold. Selye's adaptation syndrome explanation(1) has met with both acceptance and criticism. Various forms of stress are commonly used, including heat and cold, since these stimuli are easily varied quantitatively. The duration as well as the severity of the stress is undoubtedly important for adaptation may not be a simple linear function. Emphasis at

TABLE I. Comparison of Erythrocyte Number, Hemoglobin and Blood Sugar in Control Mice, after Short and Long Exposure to Cold.

	Controls			Short exposure			Long exposure		
	RBCs (M)	Hb (g %)	BS (mg %)	RBCs (M)	Hb (g %)	BS (mg %)	RBCs (M)	Hb (g %)	BS (mg %)
	7.88	13.3	185	7.98	14.0	194	8.32	13.8	190
	8.02	13.6	184	8.53	13.5	232	8.69	14.2	200
	7.53	13.4	172	8.21	12.9	208	9.23	13.9	170
	8.56	13.8	180	7.55	13.6	178	8.02	13.8	184
	8.10	13.5	177	7.69	14.0	215	8.56	13.6	182
	7.99	13.4	186	8.03	13.3	226	7.98	13.6	202
	8.32	13.4	194	7.92	14.2	198	8.88	14.0	198
	8.25	13.6	182	8.26	13.2	204	8.36	14.4	188
	8.23	13.5	175	7.89	13.8	202	8.40	14.0	172
	7.95	13.3	168	7.68	13.8	225	8.29	13.6	178
Avg	8.08	13.5	180.3	7.97	13.6	208.2*	8.47	13.9	186.4

* Only the short exposure blood sugar (208.2 mg %) is statistically significant, having a value of $P < .001$.

present is placed on the adrenal cortex(2) and thyroid(3). Controversial reports have resulted from thyroid investigation(4,5) indicating no change in level of activity of animals (rats and men) exposed to cold. The effect of cold exposure on blood sugar level has received less attention. Exposure to cold depresses the vago-insulin system while stimulating the sympathetico-adrenal system bringing about a rise in blood sugar(6). Immersion of dogs in cold water raised blood sugar(7). Prolonged exposure to low temperature results in fluid migration from blood to tissues, anhydremia(8). Others(9) believed that there is little evidence of hemoconcentration in man with acclimatization to cold. The effect of cold on glycemia level may vary with species. Thus, in the rabbit(10) no change in blood sugar with exposure to cold was found. Also the same effect was claimed for the dog (11). The same investigators(11) noted a fall in blood sugar in the fasted rabbit exposed to cold and a rise in blood sugar in the fed rabbit with cold exposure. Others(12) believe that no change in blood sugar occurs with exposure to cold unless body temperature is altered, whereupon hyperglycemia results. A seasonal variation in fasting blood sugar occurs in man(13), being higher in winter than in summer. No references were found to the effect of cold on erythrocyte number or hemoglobin but it has been shown that exposure to low temperature increases anoxic tolerance as measured by barometric decompression(14,15). Mobilization of blood sugar in short periods of time results from severe anoxia as a response to sympathetic stimulation(16,17). A similar response might result from cold stress. Anoxia causes an early elevation in blood hemoglobin which is accounted for by splenic contraction. This would also cause an apparent rise in erythrocyte number.

Material and methods. Mice of the Purdue Swiss strain were used and these were divided into 3 groups of 10 each. Group I comprised untreated controls. Group II was exposed to 5°C temperature for 15 minutes. Group III was kept in a constant temperature room of 5° for a period of 5 days. The animals were killed by decapitation with a razor blade and blood drained out by holding the animal over a watch glass. Erythrocytes were counted on a "Brite-Line" hemacytometer, hemoglobin was measured by the Sheard-Sanford method (18) using a phototelometer, and glycemia determinations were made by the Folin-Wu micro method(19).

Results. The results of the 3 groups are shown in Table I where it can be seen that the short (15 minute) exposure to cold caused a 15% rise in blood sugar while the long exposure (5 days) did not alter the blood sugar from the control level. The increase in blood sugar following short time exposure was in all likelihood a sympathetic effect resulting from cold stress and increased thermogenesis. It is interesting that blood sugar levels had returned to normal in 5 days. No long time effect of cold on either hemoglobin or erythrocyte number was seen. Possibly 5 days are not a long enough time in which to expect acclimatization to appear, or possibly no adaptive change to low temperature occurs in hemoglobin or erythrocyte number. With acclimatization to cold some hemoconcentration results(8), which could account for the increase seen with the long cold exposure. The number of erythrocytes found in this study falls within the range listed for different strains of mice(20). Hemoglobin and blood sugar levels also agree with those reported for the same strain of mice(21).

Summary. Mice were exposed to cold (5°C) for periods of 15 minutes and 5 days and the effect on erythrocyte number, hemoglobin concentration and blood sugar level determined. The only significant change occurred in the blood sugar level during the short period of cold exposure. Statistically this change had a P value of $<.001$. Possible physiological changes are discussed.

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A Plasma Extract with Erythropoietic Activity. (21066)

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Speculation still exists regarding the precise mechanism by which anoxia, the so-called fundamental erythropoietic stimulus, evokes the formation of red cells in the higher organism. Mediation through a humoral factor, termed "hemopoietine", has been proposed as one type of explanation(1-3). Erythropoietic activity in sera obtained from rabbits rendered anemic by bleeding or exposed to lowered barometric pressures was not detected in another experiment(4). However, the largest quantities administered by these workers to intact or anemic rabbits did not exceed 15 ml daily. More recent experiments (5) have tended to renew interest in this problem because of the finding that larger quantities of sera (50 ml/day), obtained from bled rabbits, induced significant rises in the numbers of peripheral red cells and reticulocytes, accompanied by increases in the percentages of nucleated erythrocytes within the bone marrow of intact rabbits.

Descriptions of the method of extraction and the properties of a protein-free fraction of plasma capable of increasing the rate of incorporation of labeled amino acids into proteins of rabbit reticulocytes have been published(6). The inference(7) that this factor and hemopoietine might be related prompted us to test the effects of this material upon the peripheral blood picture and bone marrow of the intact rat.

Materials and methods. Adult male rabbits were given daily subcutaneous injections, for a week, of one ml of a 2.5% solution of phenylhydrazine to induce intense anemia and reticulocytosis. At the end of the 7-day period, the red cell counts of the rabbits were reduced to approximately one mill/mm^3. Whole blood from these animals was collected in heparin (10 mg/50 ml blood). The plasma was brought to a pH of 5.5 by addition of 1 N HCl, boiled for 10 minutes and filtered (6). The filtrate representing 1/4 to 1/5 of the original volume of plasma was readjusted to a pH of 8.4 by addition of 1 N NaOH.* The material was placed immediately into 10 ml stoppered vials and stored in the freezing compartment of a refrigerator until used. Each of 6 normal adult female rats (150-200 g) was injected subcutaneously with 0.75 ml of this filtrate daily for 11 days. Six control rats received 0.75 ml of 1% saline solution for the same period of time. Estimations of peripheral blood values were made at intervals of 3 days for the entire experimental period and at 7-day intervals, for a period of 14 days, following cessation of treatment. Determinations were made of peripheral total red cell, total and differential white cell and total eosinophil numbers. Also included were estimations of reticulocyte percentages, hematocrit values, red cell sedimentation and fragility values, and bone marrow differentials, performed by previously described methods(8).

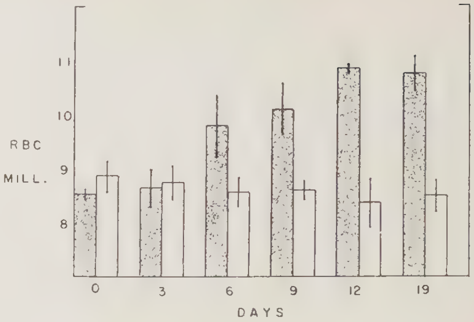

FIG. 1.

Results. Significant rises in red cell counts, hemoglobin concentrations, hematocrit values and reticulocyte percentages were induced by daily injections of the plasma extract†‡ (Figs. 1-4). The values tended to diminish after interruption of treatment. However, at 14 days after cessation of treatment (not shown in the Figs.) the red cell, hemoglobin and hematocrit values were still somewhat above the pre-injection levels.

No significant alterations were detected in the peripheral total, differential white cell and eosinophil numbers, or in the red cell sedi-

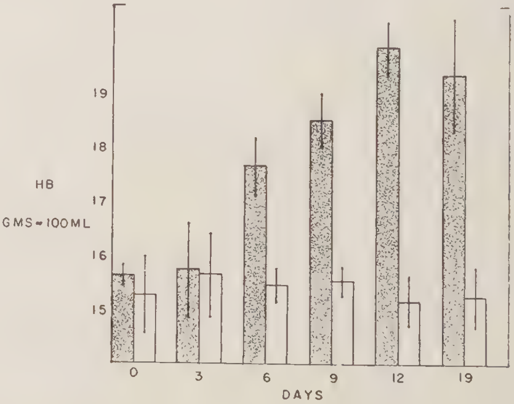

FIG. 2.

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* All extracts were provided by the Princeton Laboratories, Inc., Princeton, N. J.

† Animals injected with the extracts exhibited no apparent toxic symptoms; their body weight gains throughout the experimental period were similar to those experienced by the saline-injected controls.

‡ The more potent plasma fractions were obtained from phenylhydrazine-treated rabbits displaying the most severe anemia and the greatest reticulocytosis (in some cases 90%). The results presented in this report are based on material obtained from such intensely stimulated donor rabbits.

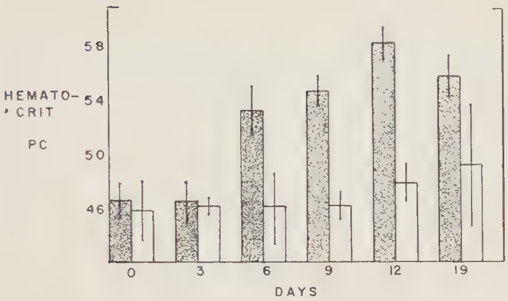

FIG. 3.

mentation and fragility values of the experimental and control animals.

The bone marrows of a group of intact rats, given 10 injections of the plasma extract, all appeared grossly reddened at the time of autopsy. Myelograms for these animals revealed approximately a 30% increase in the percentages of nucleated erythrocytes. None of the saline-injected controls exhibited these effects.

The experiments were repeated in 6 intact female rats with a lyophilized preparation of the plasma filtrate, prepared from phenylhydrazine-treated rabbits. It was reconstituted with distilled water just prior to administration. The effects of this material upon the peripheral erythrocytic values were similar to, but not as marked, as those obtained in the first series of experiments. Boiled plasma extracts obtained from untreated intact rabbits were found to be without influence upon the various red cell values in a group of 5 female rats tested.

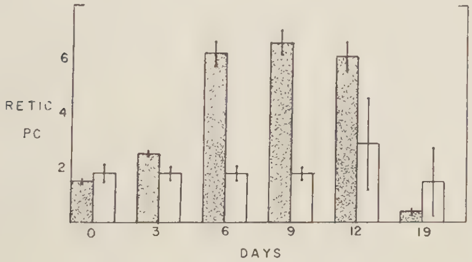

FIG. 4.

Fig. 1-4 represent alterations in red cell counts, hemoglobin concentrations, hematocrit values and reticulocyte percentages in intact rats injected daily for 11 days with 0.75 ml of an extract obtained from the plasma of phenylhydrazine-treated rabbits (stippled bars) and controls injected for same time with 0.75 ml of 1% saline solution (empty bars). Mean values are indicated. The vertical lines at the tops of the bars represent ± 1 stand. error of the mean.

Discussion. The results point clearly to the existence of a factor (or factors) in the plasma of phenylhydrazine-treated rabbits with strong erythropoietic properties when tested in the intact rat. It seems unlikely that the alterations in peripheral erythrocytic values are due to hemoconcentration effects in view of the accompanying reticulocytosis, the increases in the percentages of nucleated erythrocytes within the marrow and the absence of significant alterations in the numbers and proportions of the peripheral leucocytic elements.

Erythropoietic activity in a boiled acid extract of urine obtained from repeatedly bled rabbits has been reported(9). Inability to observe activity in similarly prepared plasma from these animals may have been due to the use of only one injection of this material. Unpublished studies in our laboratories have shown that boiled filtrates of plasma secured from rabbits subjected to repeated bleedings over a period of 3 weeks, and showing average red cell counts of 2 million/cmm and reticulocyte values of 20% at the time of sacrifice, also possess erythropoietic activity when administered daily over a 10-day period. These filtrates, however, were not as potent as those secured from the more intensely anemic phenylhydrazine-treated rabbits.

Studies are now in progress to obtain a more detailed knowledge of the chemical nature of the factor and the mechanism of its action in the intact and anemic animal. Use of this factor in a purified form could be made conceivably in patients subject to various slowly-responding or refractory anemias.

Summary. Administration to intact rats of an extract, prepared from the plasma of phenylhydrazine-treated rabbits, results in significant increases in peripheral red cell counts, hemoglobin concentrations, reticulocyte percentages, hematocrit values, and in the concentrations of nucleated erythrocytes within the marrow. The extract exerts no significant influence upon the total and differential white cell numbers or the sedimentation rates and red cell fragility values. The factor may be related to, or identical with, the cir-

culating "hemopoietine", hypothesized to be the humoral mediator of anoxia, the fundamental erythrocytogenic stimulus.

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Sensitivity Tests to Insulin in Patients with Local Skin Lesions from Insulin. (21067)

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A considerable number of diabetic individuals experience local skin reactions following the administration of insulin in the conventional manner. In previous years these were ascribed to chemical irritation arising either from the alcohol used for sterilization of syringes and needles, from the preservatives added in the manufacture of insulin or from the acidity of the earlier insulin preparations. At present, most workers consider these local manifestations as hypersensitivity to either the impurities within the insulin preparations, to the insulin protein itself or to the pancreatic protein of the species from which the hormone is derived. Recently Fabrykant and Ashe (10,10a) reported that skin changes at the site of insulin injections could be prevented by introducing the hormone into the deeper layers of the subcutaneous tissue.

The present investigation is concerned with sensitivity studies in a group of patients presenting local skin reactions while on insulin therapy in order to shed more light on the genesis of such manifestations.

Plan of study. Sixteen diabetic patients, whose local dermal reactions to insulin therapy consisted of nodules with or without erythema or stinging, were tested intradermally on the outer aspects of the arms with 0.02 cc of several insulin products in a variety of dilutions, as well as with commercial extract of beef and pork. Control tests were performed with the same quantity (0.02 cc) of Coca's diluting fluid. A group of 16 non-diabetic controls who were free from any skin affection, were similarly tested with the same materials and technic. The results were read 2, 5 and 10 minutes after the injection and were interpreted as negative, slight, moderate, marked or any combination of the last three, depending on the size of the wheal, the degree of erythema and the presence of pseudopods. For example, if the injected material did not produce a wheal greater than that from the control solution, the reaction was considered negative; if the wheal was only a little larger and a small area of erythema had developed, the reaction was termed slight; and if the wheal was more than twice the size produced by the original amount administered, with a considerable areola and the appearance of pseudopods, it was designated marked. A moderate reaction was one between these two. Some patients presented a transition from one type of reaction to another, and this was interpreted as slight-to-moderate or moderate-to-marked. We have considered as significant only the slight-to-moderate, moderate and

TABLE I. Skin Reactions of 16 Diabetic Patients with Local Response.

Product	Type of skin reaction Negative	Slight	Slight-to-moderate	Moderate	Moderate-to-marked
Regular I (full)*	0	8	6	2	0
Regular I 1:10	7	9	0	0	0
Regular I 1:100	13	3	0	0	0
Prot. zinc I (full)	1	8	4	3	0
Prot. zinc I 1:10	11	5	0	0	0
Prot. zinc I 1:100	16	0	0	0	0
N.P.H. I (full)	1	7	6	2	0
N.P.H. I 1:10	7	9	0	0	0
N.P.H. I 1:100	15	1	0	0	0
Boiled regular I (full)	0	2	10	2	2
Beef protein	11	5	0	0	0
Pork "	11	2	1	1	1

* I = insulin; (full) = full strength.

moderate-to-marked reactions. In addition, passive transfer tests were carried out with the use of sera from five patients with nodules who showed significant positive skin-test reactions to insulin. The sera were injected in the amount of 0.1 cc into 2 sites on the backs of five acceptors. Twenty-four hours later, 0.02 cc of the respective insulin preparation used by each of the 5 donors was injected into the sensitized sites as well as into 2 adjacent nonsensitized control areas.

Results. From Table I, which records the skin test in patients in whom nodules were present, it may be seen that almost identical results were obtained with the use of regular, protamine zinc and NPH insulin. Two patients who demonstrated reactions to full-strength PZI showed only a slight-to-moderate reaction to regular and NPH insulin. There were 6 patients whose reactions to regular and NPH insulin were interpreted as slight-to-moderate, and of these, 4 presented the same reaction to PZI. Dilutions of 1:10 and 1:100 of these 3 types of insulin elicited no significant skin changes. In almost all the patients tested with regular insulin previously boiled for one hour, the skin reactions were more pronounced than when there had been no boiling. Beef protein in the usual testing strength solution gave no significant reaction, and only 3 patients exhibited slight-to-moderate or marked reactions to pork protein administered in the conventional dosage.

The results recorded in the control group composed of nondiabetics were analogous to those in the diabetic patients (Table II). With full-strength regular insulin a moderate-to-marked reaction was noted in two subjects, a moderate reaction in one and a slight-to-moderate reaction in 5. One patient presented a negative reaction to regular and NPH insulin but a moderate one to PZI, and another one a slight reaction to PZI and none at all to NPH insulin. In the group as a whole the reactions to boiled regular insulin were somewhat more pronounced. Pork protein elicited but a slight response in 5 patients and a slight-to-moderate reaction in one subject whereas beef protein demonstrated but a slight response in one.

The Prausnitz-Küstner passive transfer test performed with sera from 5 patients exhibiting the most intense skin reactions when tested with insulin, evoked a negative response in each case.

Discussion. The results of the insulin-sensitivity skin tests which we have recorded in a group of patients with nodules, stinging and areas of erythema due to insulin administered by the conventional method, were no different from those obtained from a control group of nondiabetics, non-users of insulin. Boiled insulin which has been recommended as a means of avoiding allergic reactions to this hormone (11) produced a larger wheal and area of erythema than the same quantity of insulin not subjected to boiling. The negative response to beef and the occasional positive skin reactions to pork protein solutions releases these substances from the responsibility in the

TABLE II. Skin Reactions of 16 Normal Nondiabetics.

	Type of skin reaction				
Product	Negative	Slight	Slight-to-moderate	Moderate	Moderate-to-marked
Regular I (full)*	1	7	5	1	2
Regular I 1:10	11	4	0	1	0
Regular I 1:100	15	0	1	0	0
Prot. zinc I (full)	0	9	6	0	1
Prot. zinc I 1:10	14	2	0	0	0
Prot. zinc I 1:100	16	0	0	0	0
N.P.H. I (full)	3	7	3	2	1
N.P.H. I 1:10	11	4	1	0	0
N.P.H. I 1:100	15	1	0	0	0
Boiled regular I (full)	0	7	4	2	3
Beef protein	15	1	0	0	0
Pork "	10	5	1	0	0

* I = insulin; (full) = full strength.

production of local dermal reactions to insulin.

It is well known that positive skin tests do not necessarily indicate clinical sensitivity and that the typical response of the skin to injury, manifesting itself as an area of erythema with wheal formation, is the same whether it be the result of a nonspecific or of a true immunologic reaction(12).

There is a considerable mass of experimental and clinical evidence to prove that insulin can produce anaphylactic phenomena in animals and hypersensitive reactions in man. Antibodies to insulin have been reported(1,6-8,14,15), and positive results to skin test elicited with crystalline insulin as well as with modified insulin products(1-9). In a few instances passive transfer of reagins has also been accomplished(1,6-8). Since these immunologic studies were performed in animals or in humans with generalized allergic reactions to insulin (even though some of these individuals were reported to have manifested also local reactions) they have no bearing on our observations, which concern patients with skin reactions confined solely to the injected areas. It is therefore highly significant that we were unable to demonstrate any difference in the frequency and magnitude of positive skin-test reactions to various insulin products as manifested by the two groups studied namely, patients presenting local dermal reactions to insulin and the controls, who used no insulin at all.

Noteworthy too is our failure to obtain positive passive transfer tests in the five patients with a maximal response to skin testing with insulin, since in such patients one would expect positive transfer phenomena if their local reactions were truly allergic in nature. Although the group here studied may be considered too small for statistical analysis, the data clearly indicate that skin reactions to insulin of a degree sufficient to be interpreted as moderate or marked are undoubtedly due to some nonspecific mechanism and not to an antigen-antibody interaction.

Summary. 1. Sixteen diabetic patients exhibiting local skin reactions to insulin as nodules with or without erythema or stinging were tested intradermally with various insulin products, beef and pork protein. 2. Sixteen nondiabetics not using insulin were similarly tested with the same substances. 3. Passive transfer tests with the sera of 5 patients manifesting local reactions to insulin were performed. 4. Skin reactions noted in patients exhibiting local reactions to insulin were no different from those obtained in the control group of nondiabetics, non-users of insulin. 5. The Prausnitz-Küstner passive transfer phenomenon was absent in all 5 instances. 6. It is concluded that local nodules with or without stinging or erythema observed in patients receiving insulin therapy are not due to any antigen-antibody mechanism.

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Study of Ducrey's Bacillus and Recognition of a Gram-Positive Smooth Phase.* (21068)

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In the current and generally accepted classification system, *H. ducreyi* appears to have a rather definite taxonomic position in the genus Hemophilus(1). A review of the published studies lends little support to this view, and reveals that the accumulated knowledge of this bacterium is exceedingly limited (2). The lack of basic information appears to be due primarily to the difficulties which have attended the cultivation of Ducrey's bacillus. *H. ducreyi* is the recognized cause of chancroid. Most investigators agreed that the causative microorganism is extremely difficult, if not impossible, to isolate from primary chancroidal lesions. Primary lesions are invariably contaminated with a variety of bacterial species and this together with the fastidious growth requirements of *H. ducreyi* appear to make identification of this organism impossible by present-day procedures(3). The well-known characteristics of Ducrey's bacillus seem to have resulted from studies of cultures which were obtained from bubo material. The successful isolation of *H. ducreyi* from aspirated bubo fluid has been reported on numerous occasions(4,5). In all investigations the importance of incorporating whole blood in culture media has been emphasized. It has been determined that a growth factor necessary for the Hemophilus group is also required for *H. ducreyi* cultivation(6). Previous bacteriologic procedures have not permitted detailed characterization of Ducrey's bacillus in respect to colonial morphology, or to its physiologic or serologic behavior. The bacteriologist, therefore, with limited means of identification, can offer little assistance in the diagnosis of chancroid as a disease entity. The diagnosis of chancroid, therefore, is dependent upon clinical impressions which are not yet clearly defined. The development of new methods and procedures for the cultivation and identification of Ducrey's bacillus could offer a partial solution to this diagnostic problem. The successful accomplishment of these objectives should eventually help to explain some of the unanswered questions pertaining to epidemiologic and immunologic aspects of the disease.

The present report deals with a description of our experimental methods and procedures applied to the study of Ducrey's bacillus.

Experimental. Stock *H. ducreyi* cultures were received from Dr. R. B. Dienst, Medical College of Georgia; Dr. J. D. Thayer, Venereal Disease Experimental Laboratory, Chapel Hill, N. C. and from the Lederle Laboratories.

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* This work was supported in part by the Medical Research and Development Board, Office of the Surgeon General, Department of the Army.

While it was not possible to obtain complete histories of all of the cultures it is assumed that they were originally isolated from bubo pus. The oldest culture of this group, strain "Hunt", was isolated by Dr. G. A. Hunt in 1935. The youngest culture, strain ATC, was listed as having been isolated in or about 1947. Most of the old cultures were received in the dried state and were reconstituted or sub-cultured to sterile defibrinated rabbit blood-infusion agar slants according to the method of Sanderson and Greenblatt(5). Cultures were incubated at 35°C and were examined microscopically at 24-hour intervals. All of the old stock cultures behaved in a manner which might be described as classical, that is when once growth had become established by any one strain, subsequent sub-cultures usually demonstrated good growth after a 24-hour incubation period. Microscopic examinations demonstrated typical gram-negative long-chained streptobacilli. Rabbit inoculation experiments with these cultures were invariably negative. In an effort to determine colonial characteristics the stock cultures were plated on Eugon Agar (BBL) plus 15% sterile rabbit blood. Both the candle and the Brewer anaerobic jar methods were used for petri plate incubation. Growth and colonial morphology was approximately the same by either method. The colony size of any one strain usually varied between 0.5-1.0 mm in diameter after 72 hours incubation. By reflected light, colonies showed a distinct roughness in surface texture and in some cases the margins were uneven. There was a decided tendency for most colonies to crumble when touched with the inoculating needle, some would slide along the surface of the agar when attempts were made to "pick" them. Many colonies were apparently made up of non-viable bacteria as evidenced by growth failure of numerous transplants. All our attempts to grow old stock cultures in media other than those rich in sterile blood were unsuccessful.

In order that virulent and non-virulent strains might be compared, isolation of new cultures was attempted. Three cultural methods were used, Eugon Agar (BBL) plus 15% sterile defibrinated rabbit blood plates were streaked with freshly aspirated bubo pus and incubated at 35°C in both candle and Brewer anaerobic jars; infusion agar slants overlayed with sterile defibrinated rabbit blood were inoculated according to the method of Sanderson and Greenblatt(5). Plates were examined both macro and microscopically after 72 hours. Blood tube cultures were examined microscopically at 24-hour intervals. In the 3 cases reported here the cultural results were identical; growth occurred only on plates which had been incubated under anaerobic conditions. Colonies from each of the three cases were similar but in no way resembled the colonies previously described for old stock cultures. Colonies obtained from the bubo material measured from 1-3 mm in diameter, were smooth, white, and dew-drop in appearance, and almost opaque by reflected light. Most of the colonies were surrounded by a distinct zone of hemolysis. The staining and morphologic appearance of the bacteria composing these newly isolated colonies was entirely dissimilar to those described as occurring in the old stock cultures. All of the new cultures contained gram-positive rods of various sizes, the predominant type being long and slender, the dimensional proportion being 8-10 times as long as their width. In some cases more deeply staining granules were present within the rods. There was little if any evidence of chain formation; a few diplobacilli could be seen in the average microscopic field. The majority of the organisms were seen as scattered individual rods or were assembled in irregular groups.

When our new cultures were transplanted and maintained in rabbit blood a rather rapid variation in colony type, bacterial morphology and staining characteristics was observed. Five to eight weekly transfers were usually sufficient to complete these changes. After this period of time the new cultures were similar in every respect to the old stock cultures previously described. We have been able to maintain the new strains without change by frequently subculturing under anaerobic conditions. Carrying media are being investigated, but at present all of the stock cultures, new and old, have been preserved by the freeze-dry process.

Several investigators have reported the production of chancroidal lesions in rabbits(7,8). From their descriptions it is apparent that success was dependent upon the use of freshly-isolated bubo cultures.

Rabbit virulence studies were conducted using our new strains. Best results were obtained when 0.2-0.3 ml of a 72-hour rabbit blood culture was inoculated intradermally. Most of the inoculations have been made into the shaved abdominal skin. Other inoculation sites appear to be quite as satisfactory. Virulent cultures usually demonstrated evidence of infection within 24 hours.

The lesion commonly had the appearance of an erythematous nodule occurring superficially in the epidermis at the site of injection. There was slight induration palpable beneath the lesion. The area of surrounding erythema extended approximately 2 centimeters beyond the nodule. Lesions appeared somewhat edematous and were larger than the original bleb produced by the inoculum. There was at times slight scaling of the surface of the lesion. With the passage of 4-5 days nodular lesions gradually increased in size to about 2 cm and take on the characteristics of a subcutaneous abscess, together with a decreasing surrounding erythema. There was a palpable softening of the contents of the abscess and after 5 days was frequently ulcerated and exuded a gummy purulent exudate. The ulcer appearing at the apex of the abscess did not generally enlarge beyond the limits of the nodule delineated prior to its ulceration. After rupture the abscess gradually discharges its contents, the ulceration produced heals slowly but spontaneously over a period of about 2 weeks leaving a slight scar.

Viable Ducrey's bacilli were always demonstrable in these lesions before ulceration had occurred. On the day following rupture of the abscess we have usually been unsuccessful in our attempts to recover the causative bacterium.

Discussion. Our comparative study of old and freshly-isolated cultures of Ducrey's bacillus indicates that past descriptions of this species have dealt for the most part with a rough non-pathogenic variant. Our observations correspond to the well-known smooth (S)—rough (R) variations which have been observed in other bacterial groups. Our observations appear unique in respect to the radical nature of the variation. The difference is so great in the case of Ducrey's bacillus that the parent or "S" form would necessarily be classified in an entirely different family than the variant "R" form. It seems likely that the previous well-known and described characteristics of *H. ducreyi* strains are those of a "loss variant". The loss in this case appears to be associated with both antigenic and physiologic behavior. In our investigations which are in progress, we have discovered only slight antigenic relationships between the "S" parents and "R" variants. If growth factors found in whole blood are indeed necessary for the cultivation of either the "S" or the "R" forms, their effects are much more pronounced in connection with the "R" forms. We believe that the principal part played by whole blood in past experiments has been its ability to produce a favorable anaerobic environment. In concurrent experiments excellent growth of various anaerobic species in whole blood tubes without the use of special seals or other anaerobic equipment has been observed. The results of our observations require a reclassification of Ducrey's bacillus, the establishment of its exact taxonomic position however awaits more extensive investigation of its physiologic and antigenic behavior. With the information now known progress may be expected pertaining to immunologic and epidemiologic aspects of the disease chancroid.

Summary. 1. Stock strains of Ducrey's bacillus obtained from various sources have been found to conform to classical and current descriptions of *H. ducreyi.* 2. Comparison of stock "R" strains with freshly isolated anaerobic cultures, indicates that the "S" form of this species has not been previously recognized. 3. Our experiments indicated that blood is not required for growth of the "S" form and that its use leads to rather rapid production of the classical "R" variant currently classified as *H. ducreyi.*

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Weight, Ascorbic Acid and Cholesterol Changes in Adrenals of Pyridoxine-Deficient Adult Male Rat. (21069)

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Impaired adrenal functions in the pyridoxine-deficient rat have been reported by several workers employing histochemical methods(1), water diuresis tests(2), survival rates during cold exposure(3), and lymphopenic responses (4).

In this study an endeavor was made to measure the functional capacities of the adrenal of the pyridoxine-deficient rat by the responses to two stimuli, a) the administration of adrenocorticotropic hormone (ACTH) in hypophysectomized animals, and b) the exposure of intact animals to hypoxia. Depletion of the ascorbic acid and cholesterol content of the adrenal of the normal rat follows the administration of ACTH(5) or subjection to hypoxia(6,7). This depletion of adrenal ascorbic acid and cholesterol has been widely employed for the assay of ACTH preparations as well as for an index of the functional capacities of the adrenal gland.

Procedure. Long-Evans strain male rats were employed for all experiments. At 103 days of age the animals were placed on a diet of 24% casein, 10% hydrogenated vegetable oil, 4% salt mix(8) and 62% sucrose. A vitamin mixture[†] was fed 3 times weekly and pyridoxine was omitted from the mixture given the deficient group. Desoxypyridoxine, 30 mg/kg diet, was added directly to the ration of the deficient group. Three groups were studied, deficient, pair weighed and *ad libitum.* During the 8th week on the diet, 60 animals were subjected to 24 hours of fasting. For the last 20 of these hours the rats were placed in individual glass chambers, 30 of which were maintained at a pressure of 349 mm of Hg equivalent to 20,000 ft altitude and the remainder at sea level pressure. All animals received water *ad libitum* through a tube entering each glass jar. *Hypophysectomy* operations were performed on another group of 60 animals at the end of the 7th week on the regimen. For 2 days after the operation they received daily injections of 2 cc of 20% glucose intraperitoneally and their drinking water contained 10 mg % aureomycin and 5% glucose. The glucose intake was then discontinued and all animals were fasted for a total period of 18 hours. Half of these animals were injected during the fasting period with ACTH (Armours, ACTHAR, lot L5072) dissolved in 0.9% NaCl. The remaining animals received equal volumes of 0.9% NaCl.

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[†] Water soluble vitamins were dissolved in 20% aqueous ethanol and fed 3 times weekly in individual cups. This supplied each rat daily the following amounts in mg; thiamine hydrochloride, riboflavin, pyridoxine and folic acid, .02 each, calcium pantothenate and paraminobenzoic acid, .10 each, niacin amide, .07, inositol, 2.5, biotin, .002, and choline, 5.0. Vit. A and D and mixed tocopherols were dissolved in vegetable oil and were supplied separately in amounts of 1000 I.U., and 100 I.U., and 3 mg/rat/week.

Three doses of ACTH were given, 1 I.U., 7 I.U., and 2 I.U. per 100 g body weight on the zero, 9th and 16th hours respectively. Autopsies were done 2 hours after the last injection using sodium barbital anaesthesia. After exsanguination from the heart, each adrenal was weighed separately. One was homogenized with 5% trichloroacetic acid and the other with a 50:50 mixture of acetone and absolute alcohol. *Chemical methods.* The total adrenal cholesterol was determined by the Schoenheimer and Sperry method(9). The Roe and Keuther method(10) was employed for the determination of adrenal ascorbic acid.

Results. Intact fasted adults at sea level or under hypoxia. The adrenal ascorbic acid contents and adrenal weights at sea level in the deficient, pair weighed and *ad libitum* groups were equal. Hypoxia caused no significant depletion of ascorbic acid or increase in the adrenal weights of any group (Table I). It is surprising that adrenal weight changes were not seen since Langley and Clarke(11) found that maximum adrenal weight increases occurred within the first 2 days during hypoxia exposure. The concentration of adrenal cholesterol in the deficient and *ad libitum* groups at sea level did not differ but both groups had significantly lower values than did the pair weighed group ($p < .05$ and $< .01$, respectively). Significant cholesterol depletion ($p < .01$) followed hypoxia exposure, 23% in the pair weighed group and 43% in the *ad libitum* group. In marked contrast, the cholesterol depletion, by 11%, in the deficient group was not significant. Tepperman and co-workers(7) have reported significant cholesterol depletion without ascorbic acid depletion in normal rats exposed to 25000 ft altitude for 5 hours.

Hypophysectomized adults, saline or ACTH treated. In the 3 saline treated groups the adrenal weights and ascorbic acid concentrations did not differ (Table I). The cholesterol concentrations in the saline treated deficient and pair weighed as well as the deficient and *ad libitum* groups were equal, but the pair weighed group had a higher cholesterol value ($p < .01$) than the latter group. All groups treated with ACTH as compared with their respective saline injected groups showed a distinct decrease ($p < .01$) in adrenal ascorbic acid, 31% to 45%, a marked increase, 20 to 25% in the adrenal weights ($p < .01$) but no decrease in adrenal cholesterol. The adrenal cholesterol in the ACTH treated *ad libitum* group was even increased by 18% ($p < .01$) above its saline value. The reason for the latter finding is not apparent.

TABLE I. Weights, Ascorbic Acid and Cholesterol Contents of Adrenals of Intact and Hypophysectomized Pyridoxine-Deficient Rats. (8 to 12 rats in each group.)

Conditions	Adrenal wt, mg	Adrenal ascorbic acid, mg %	Adrenal cholesterol, %
	I. Intact animals		
At sea level			
Deficient	46.7±2.4*	410±19	2.19±.14
Pair weighed	42.8±1.4	415±21	2.65±.20
Ad libitum	45.4±1.9	419±12	1.95±.13
At 20000 ft			
Deficient	50.6±2.9	465±26	1.96±.12
Pair weighed	40.4±2.7	464±24	2.03±.11
Ad libitum	49.6±1.9	360±41	1.01±.11
	II. Hypophysectomized animals		
Saline inj.			
Deficient	34.5±1.8	336±19	2.56±.14
Pair weighed	32.7±1.6	307±11	2.64±.12
Ad libitum	32.2±1.3	336±18	2.24±.07
ACTH inj.			
Deficient	43.4±2.5	183±15	2.59±.19
Pair weighed	39.3±1.8	215± 8	2.63±.25
Ad libitum	38.7±1.3	184± 7	2.65±.12

* In all cases mean and stand. error.

Discussion. The failure of adrenal cholesterol depletion during hypoxia is clearly due to the lack of pyridoxine apart from inanition. Several factors may have caused the observed impairment. Failure of release of sufficiently increased quantities of ACTH or adrenal cortical steroid hormones or both may have occurred. Butler and Morgan(12) concluded that the pyridoxine-deficient intact rat did liberate augmented amounts of ACTH and adrenal 11-oxysteroid hormones under hypoxia since maximum eosinopenic responses occurred. But in the absence of pyridoxine the increased release of these hormones may have been inadequate for chemical evidence of adrenal cholesterol depletion.

The adrenal of the pyridoxine-deficient, hypophysectomized animal displayed no impairment in ascorbic acid depletion and adrenal weight increase following ACTH administration. A number of workers(13-15) have reported a lack of correlation in the biological activities of various ACTH preparations. These findings have led to the recent viewpoint(15) that there are several adrenocorticotropic hormones having different biological activities. Until this question is resolved, conclusions are not justified concerning the pituitary or adrenal origin of the impairment in adrenal cholesterol depletion observed in the pyridoxine-deficient rat. One may not at present regard the depletion of ascorbic acid and cholesterol of the adrenal and its weight increase as comparable biological activities measuring the same functional activity of the gland. Better understanding is needed of the physiological processes involved in the production of adrenal cholesterol and its relationship to hormone production.

Summary. Two days after hypophysectomy, the pyridoxine-deficient rat treated with ACTH had the same amount of adrenal ascorbic acid depletion and adrenal weight increase as did the pair weighed and *ad libitum* controls but like these controls no decrease in adrenal cholesterol. When intact animals were subjected to hypoxia, however, the pyridoxine-deficient group, although able to maintain adrenal weights and adrenal ascorbic acid content as did the pair weighed and *ad libitum* groups, clearly failed to show the adrenal cholesterol depletion characteristic of the latter groups. The depletion of adrenal cholesterol is apparently not controlled by the same mechanism involved in adrenal hypertrophy and adrenal ascorbic acid depletion.

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Carcinogenic Activity of Radioactive Phosphorus.* (21070)

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The development of malignant tumors has been described in rats which survived a dose of radioactive phosphorus in the LD_{50} range, *i.e.*, 4.5 μc per gram body weight(1). The present study indicates that even when substantially smaller doses are used, P^{32} is still a potent carcinogenic agent.

Method. A total of 123 adult white male rats of the Wistar strain, weighing about 150 g each, obtained from Carworth Farms, were used. These were divided into 6 groups in which each animal received a single dose of 0.25, 0.5, 1.0, 2.0, 3.0 or 3.5 μc of P^{32} per

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TABLE I. Incidence of Malignant Tumors.

Dose of P^{32} (μc/g body wt)	Original No. of rats	Rats alive after 8 mo No.	Life span (mo)	% incidence of malignant tumors
.25	25	21	11–35	0
.5	25	22	11–31	0
1.0	20	19	13–24	32
2.0	16	13	11–30	31
3.0	20	18	9–32	22
3.5	17	16	9–12	50

gram body weight respectively. The rats were housed individually in an air-conditioned unit permitting constant temperature and humidity. Radioactive material was administered by the intraperitoneal route. After 8 months periodic x-rays were taken of the entire skeleton of most animals and a routine x-ray of the skeleton was made of every rat at the time of death.

Results. Table I shows the number of animals in each group originally, the number alive after 8 months and their subsequent life span, and the incidence of malignant neoplasms. In calculating incidence those rats in the 0.25, 0.5, 1.0 and 2.0 μc groups which died before 8 months and those in the 3.0 and 3.5 μc groups which died before 5 months, *i.e.*, considerably before the latent period for tumor development, were not included. Also lymphosarcoma, which occurred in one animal in each of the 0.25, 1, 2 and 3.5 μc groups was omitted since this tumor has occasionally been observed to occur spontaneously in the C. F. Wistar strain.

Outside of one rat with lymphosarcoma, the 43 animals receiving the 0.25 or 0.5 μc dose of P^{32} did not develop tumors. In the groups where dosage ranged from 1.0 to 3.5 μc of P^{32}, neoplasms occurred in from 22 to 50% of animals. The greatest frequency was in rats which received the highest dose of radioactive phosphorus. However, among the 1, 2 and 3 μc groups there was no indication that the incidence of malignancy decreased with dosage in the manner described for radioactive strontium(2).

Table II gives the number of rats in each group with neoplasm, the latent period for tumor development, and the types of neoplasm. Only 2 types were observed, osteogenic sarcoma and squamous cell carcinoma. The former was more common and occurred in a total of 18 animals with the following locations: femur 7, spine 4, tibia 3, jaw 3, and humerus 1. There were metastases in 10 of the 18 rats, usually to lungs and regional lymph nodes. Six animals developed an extensive ulcerating squamous cell carcinoma involving one side of the face with no metastasis. Except for one rat injected with 1 μc of P^{32} per gram body weight, the squamous cell tumors occurred in animals which received the highest dose of radioactive phosphorus, *i.e.*, 3.5 μc per gram body weight.

Discussion. The overall incidence of neoplasms produced by smaller amounts of P^{32} was less than that previously obtained with very high doses of the isotope(1). In addition the latent period of tumor development appeared to be lengthened when the dose of P^{32} was lowered. Thus the mean time of development was 19, 14 and 10.5 months respectively for animals given 1, 2 and 3 μc of P^{32} per gram body weight. This observation is limited by the fact that these periods represent detection of the neoplasm rather than the exact time of origin.

The two types of malignant tumor induced in this study were the same as those which resulted from large doses of P^{32}. Osteogenic sarcoma was the most common neoplasm. This would be anticipated since P^{32} is deposited heavily in the skeleton and causes radiation osteitis which is a precursor to malignant change. The second variety of neoplasm resulting from P^{32} was squamous cell carcinoma of the face. This appeared to arise below the skin rather than from the surface and to invade the subcutaneous soft tissues, bones of the face and orbit. While the exact origin has not been determined, the probable source is epithelium closely approximated to bone, *i.e.*, lining of accessory nasal sinuses, nasopharynx, and roof of mouth, and presumably irradiated from this site.

The validity of our data obviously depends on the spontaneous incidence in rats of osteogenic sarcoma and cancer of the face. As far as is known, these tumors are rare among normal animals of the Wistar strain. In 58 such rats followed through their lifetime, we

TABLE II. Latent Period and Types of Neoplasm.

Dose of P^{32} (μc/g body wt)	No. of rats with neoplasm	Latent period (mo)		Types of neoplasm	
		Range	Mean	Osteogenic sarcoma	Squamous cell carcinoma
1.0	6	12 – 25	19	5	1
2.0	4	12 – 15	14	4	0
3.0	4	8 – 14	10.5	4	0
3.5	8	7.5 – 13	10	5*	5*

* Two rats in the 3.5 μc group had both osteogenic sarcoma and squamous cell carcinoma.

found one instance of osteogenic sarcoma and none of squamous cell carcinoma(3). Ratcliffe(4) reported that about 5% of rats in a Wistar stock colony developed neoplasm and that among the 75 tumor-bearing animals comprising this 5% there was one osteogenic sarcoma and one carcinoma of the skin. The latter arose from the tip of the snout. A very low overall incidence of these two malignant neoplasms has also been described in other strains of the rat(4,5).

In this study the smallest dose of P^{32} which induced tumors was 1 μc/gram body weight and this corresponds to 70 mc for a 70 kg human, *i.e.,* about 5 times the usual therapeutic dose. Amounts of 0.5 and 0.25 μc of P^{32} per gram, which approach the average therapeutic dose in man, did not prove carcinogenic. From the experimental standpoint P^{32} can be used as an effective and relatively simple method of inducing malignant neoplasms. The optimum dosage is probably from 1 to 3 μc per gram body weight. Larger amounts will also be carcinogenic but the early mortality from radiation is potentially greater and the life span of the surviving animals shorter.

Summary. 1. Single doses of radioactive phosphorus, administered internally to rats in amounts of from 1 to 3.5 μc/gram body weight, proved carcinogenic. Two types of malignant neoplasms were produced, *i.e.,* osteogenic sarcoma and carcinoma of the face. 2. The incidence of neoplasms ranged from 22 to 50%. The latent period of tumor development was lengthened with smaller doses of P^{32}. 3. Amounts of P^{32} less than 1 μc/gram body weight failed to induce neoplasms.

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Chemical Inhibition of Cell Division of *Escherichia coli* by Diazo Compounds and Antagonism by Tyrosine. (21071)

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Studies with *Escherichia coli* have shown that certain organic chemicals, such as nitrogen mustards, triethylenemelamine, diazouracil, and other diazo compounds produce the same morphological effects, *i.e.,* the inhibition of multiplication without inhibition of culture growth or mass, as alpha or X-irradiation(1,2) and produce similar biochemical transformations(3). Since 5-diazouracil had proved to be a highly effective cell-division inhibitor and since both 5-aminouracil and

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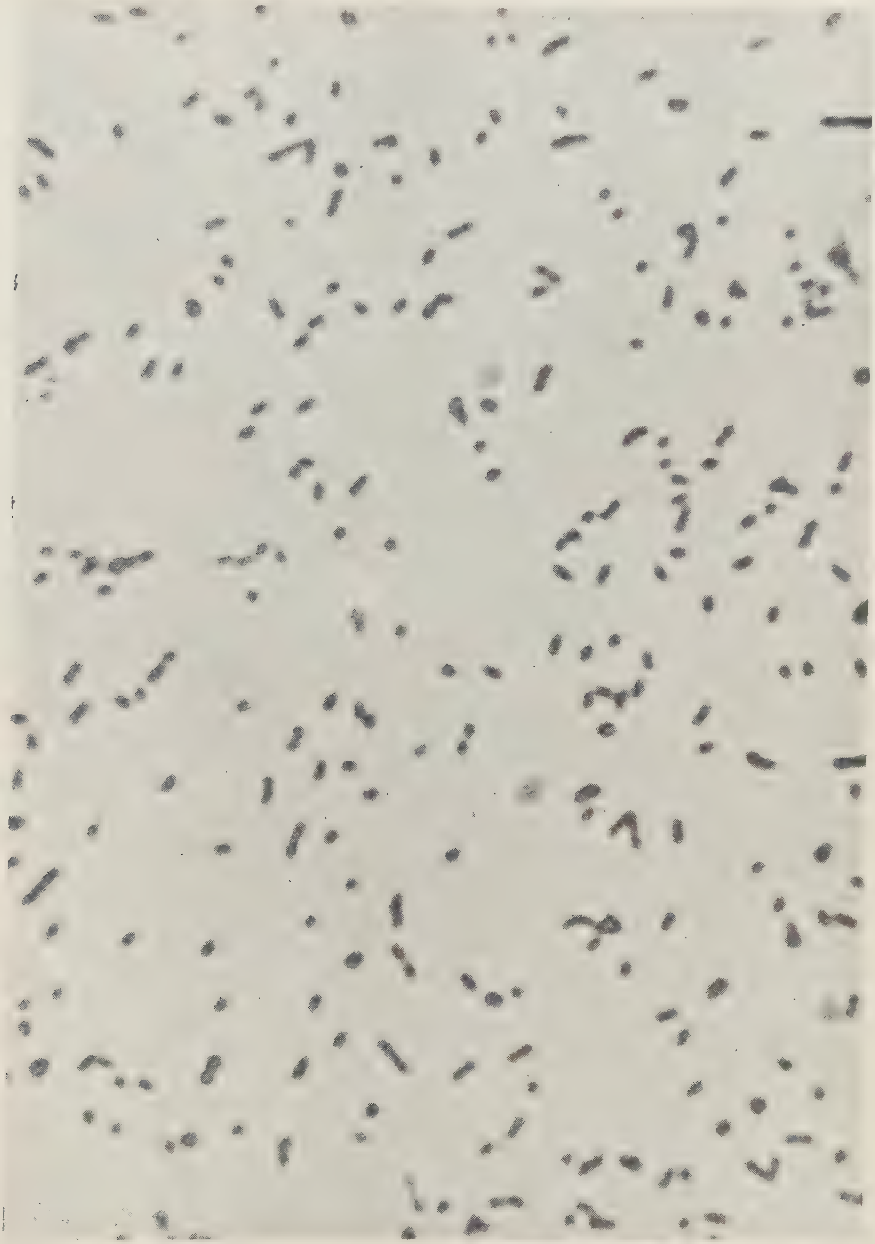

FIG. 1. Control untreated *E. coli* culture in the logarithmic phases of growth and division; cell count, 2×10^9 cells/ml; culture weight, 0.78 mg/ml (×475).

uracil were inactive compounds, diazouracil was taken as a model for two types of additional studies: a) to test whether the cell division inhibitory activity was due to the diazo group of the molecule; and b) to study the relationship of the inhibition effected by diazouracil to important biochemical metabolites.

The present paper deals with the effects of a variety of aliphatic and aromatic diazo compounds on the division and growth of *E. coli* and with the antagonism of diazouracil division-inhibition by tyrosine.

Methods. Growth and testing conditions

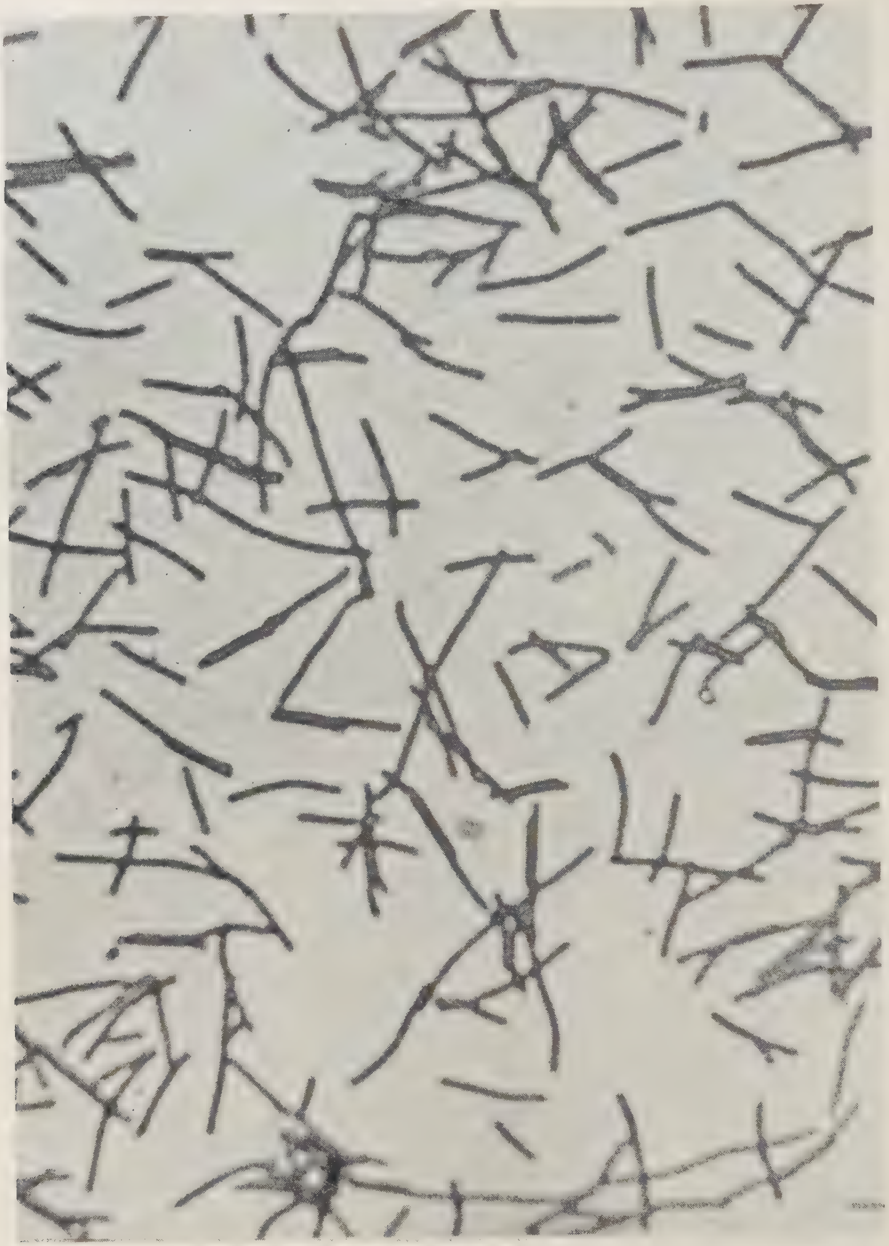

FIG. 2. Experimental *E. coli* culture, after logarithmic growth with 5-diazouracil for 1½ hr; cell count, 5×10^8 cells/ml; culture weight, 0.78 mg/ml (×475).

with *E. coli* have been described previously (1). Diazouracil at concentrations of 0.3 μg/ml completely inhibits division of *E. coli* without decreasing culture mass, producing morphologically uniform cultures of filaments which are 3 to 5 times the size of normal cells as shown in Fig. 1 and 2. In all assays controls consisted of cultures with and without diazouracil. In experiments to antagonize the division-inhibition, simultaneous additions of the inhibitor and the antagonizing agent were made. Growth measurements were based on culture dry weights; cell counts were made to determine the effect upon division. Two methods of diazotization of primary aromatic amines were used; for amines which readily formed diazonium salts in aqueous solutions, the conventional method of diazotization was performed, *i.e.*, sodium nitrite was added to an equivalent quantity of the amine in an excess (3 eq) of mineral acid at 0-5°C(4). With difficultly-diazotizable amines concentrated nitric acid was added to the amine followed by the addition of an equivalent amount of a reducing agent, *e.g.* sodium metabisulfite(4). Spot tests by coupling the diazotization mixture with α-naphthol or α-naphthylamine were used to check qualitatively the effectiveness of diazotization(5).

Results. Cell division inhibition studies. The simplest aliphatic diazo compound, gaseous diazomethane, which was tested numerous times by various means, was shown, but only sporadically, to inhibit cell division of *E. coli.* Excessive amounts of diazomethane did inhibit growth, presumably by reacting with the glucose making it unavailable as a substrate for the culture, as shown by the failure of treated media to form a precipitate with 2,4-dinitrophenylhydrazine. The actual dose of diazomethane required to inhibit division could not be determined because of experimental difficulties in administering the gas to the cultures. However, the precursor of diazomethane, N-nitrosomethylurethane, at concentrations of 0.3 μg/ml promoted the formation of long filaments which were 3 to 5 times the size of the control cells. Other aliphatic diazo compounds, such as ethyl diazoacetate, diazoacetone, and diazoacetophenone, were either ineffective on division processes or at concentrations above 3 mg/ml were inhibitory. Diazotized aniline, 2-aminofluorene, and N,N-dimethyl-p-phenylene-diamine sulfate at concentrations of 30-10 μg/ml inhibit cell division of *E. coli.* p-Nitroaniline, 2,6-diaminopurine and p-aminobenzenesulfonamide when diazotized produced a slight inhibition of the organism. Certain other aromatic amines, pyrimidines, and purines were ineffective division inhibitors or were extremely toxic to growth.

Antagonism of diazouracil inhibition. Although the absence of an effect on the total culture mass with 0.3 μg diazouracil/ml seemed to preclude the concept that division inhibition resulted from a block in a major energy transfer system, adenosine triphosphate (ATP), adenylic acid, and diphosphopyridine nucleotide were tested for antagonistic activity. None of these compounds at concentrations of 5 × 10^{-4} M or less alleviated the division block, although an exogenous source of ATP stimulated total culture growth by as much as 50%.

The additions of the intermediates of the glycolytic and the tricarboxylic acid cycles to diazouracil-inhibited cultures were without effect on the cell size of the bacteria. Yeast extract (Difco), Bacto-peptone, and casamino acids (hydrolyzed casein) were all observed to have slight antagonistic effects on inhibited cells and were observed to increase culture growth by 30 to 50%. These effects, however, were not striking and were obtained only at high concentrations (1-3 mg/ml).

L-Tyrosine at a concentration of 30 μg/ml completely antagonized the inhibition by diazouracil resulting in cells which were morphologically normal in appearance as shown in Fig. 3. 3,4-Dihydroxyphenylalanine, L-phenylalanine, L-serine, D- and L-tryptophans, tyramine, L-alanine, phenol, 3,5-diiodotyrosine, L-thyroxine, p-hydroxybenzoic, L-phenyllactic acid, p-hydroxyphenylglycine, and other amino acids were ineffective.

Such inhibition experiments, however, do not rule out the possibility that the antagonizing action of tyrosine to diazouracil is due merely to titrating out the inhibitor or to chemical inactivation of the inhibitor. Consequently, three additional types of experiments were performed: a) various mixtures of diazouracil and tyrosine were incubated at 37°C at pH 7 and pH 2. One-dimensional chromatograms in butanol-acetic acid-water solvent of these mixtures were compared with chromatograms of tyrosine. In all cases the R_f values and the intensity of the ninhydrin developed spots of the mixtures were identical with those of appropriate tyrosine controls; b) the ultraviolet absorption spectra of incubated mixtures of tyrosine and diazouracil showed no shifts in absorption maxima from the spectra of tyrosine and diazouracil alone; c) the spectrophotometric de-

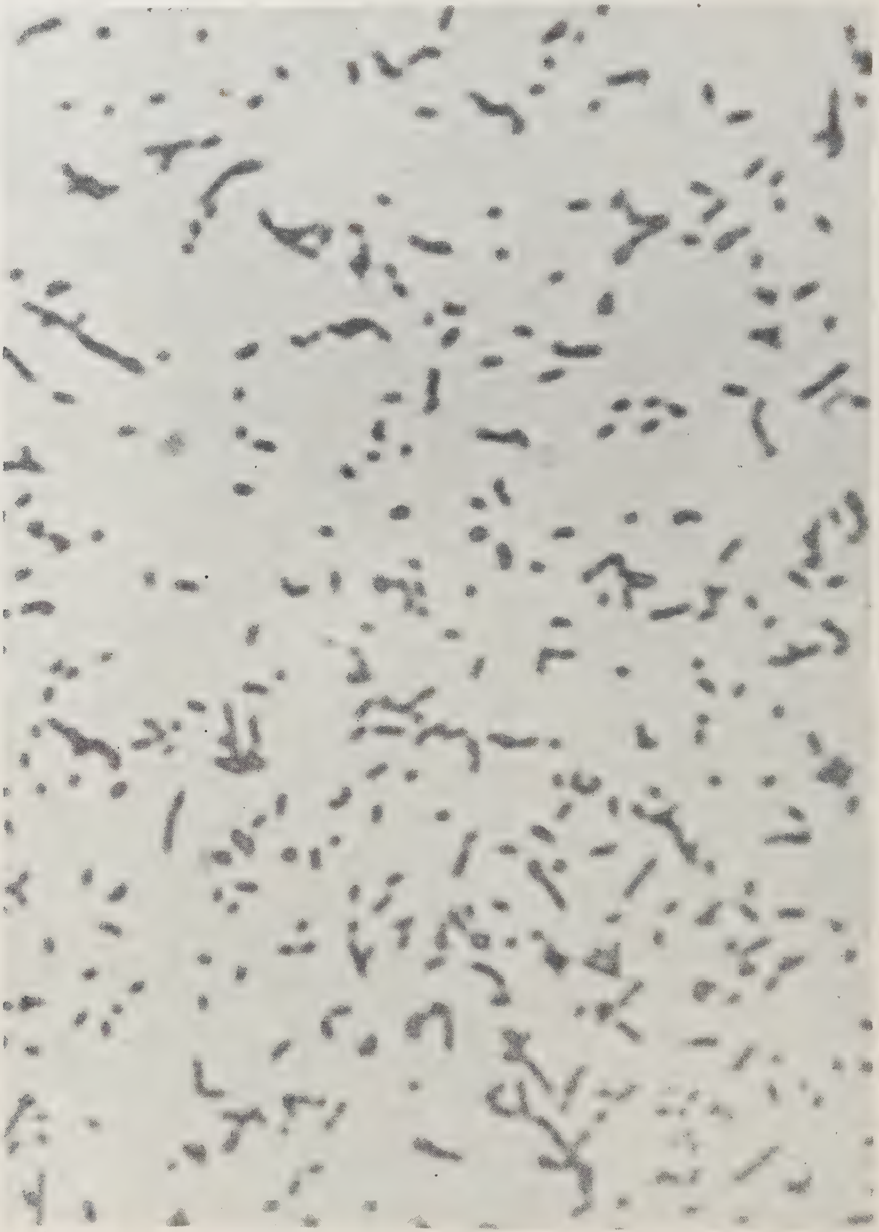

FIG. 3. Experimental *E. coli* culture treated with 5-diazouracil and with tyrosine; cell count, 1.5×10^9 cells/ml; culture weight, 0.78 mg/ml (×475).

termination at 540 mμ of diazouracil and incubated diazouracil-tyrosine mixtures after coupling with α-naphthylamine showed no decrease in the diazouracil concentration. The evidence from these experiments indicates that there is no interaction between diazouracil and tyrosine; consequently, the antagonism of diazouracil inhibition by tyrosine is not a result of the destruction of diazouracil.

In contrast to these observations it can be shown that sodium hydrosulfite and ascorbic acid, but not dehydroascorbic acid, antagonize diazouracil inhibition by inactivating the diazouracil. Diazouracil activity can also be destroyed by pretreatment with alkali.

Discussion. While all the synthesized diazo compounds were not effective cell-division inhibitors, the structures of the effective compounds are so dissimilar that the activity of these compounds as inhibitors appears to be due to a functional group specificity rather than a total structural specificity. Such an hypothesis would indicate that the activity of diazouracil is due to the diazo portion of the molecule rather than to the uracil moiety. Additional work is being conducted in an attempt to determine whether diazo groups at positions other than the 5-position of the pyrimidine molecule are as effective in inhibiting cell division.

Investigation of the relationship between cell-division inhibition by diazouracil and tyrosine antagonism is being continued in an attempt to determine the role of tyrosine in cell division. It is apparent that inhibition of cell division in these experiments was not a result of a block in reactions dependent on high-energy phosphate. Other work in this laboratory has shown that there are no significant changes in the phosphorus fractions of these inhibited *E. coli* systems(3). It may be that tyrosine and/or aromatic amino acid metabolism are involved in the inhibition of cell division.

Summary. Studies were made to determine the specificity of the chemical inhibition of division of *Escherichia coli* and to determine the relationship of division inhibition to important biochemical metabolites. It was found that the cell-division process of *E. coli* could be specifically inhibited, without an accompanying inhibition of culture growth, by diazouracil and by other diazonium compounds, and it is suggested that the diazo group on the compounds is responsible for the inhibition. The inhibition of division by diazouracil was antagonized by tyrosine, and it was shown that the antagonism can not be attributed to a destruction of the inhibitor by tyrosine. Other amino acids, intermediate in the glycolytic and tricarboxylic acid cycles, and energy-rich phosphorus compounds were incapable of antagonizing diazouracil-inhibited cultures.

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Role of Antral Exclusion in Development of Peptic Stomal Ulcer.* (21072)

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The importance of the chemical phase of gastric secretion is well known. This phase is mediated through the production of the hormone gastrin in the antrum of the stomach when food or its digestion products come in contact with the antrum. Dragstedt *et al.* (1,2) have shown that transplantation of the antrum to the colon produces a profound and sustained increase in the secretion of hydrochloric acid from an isolated gastric pouch and also that it causes the development of peptic stomal ulcers in a high proportion of dogs. More than 30 years ago Koennecke(3) had shown that placement of the antrum upon the distal ileum of dogs invited stomal peptic ulcers. Recently, Sauvage *et al.*(4) reported a series of experiments which suggested that the antrum was the most important single factor in the control of gastric secretion. However, all of the experiments reported by Sauv-

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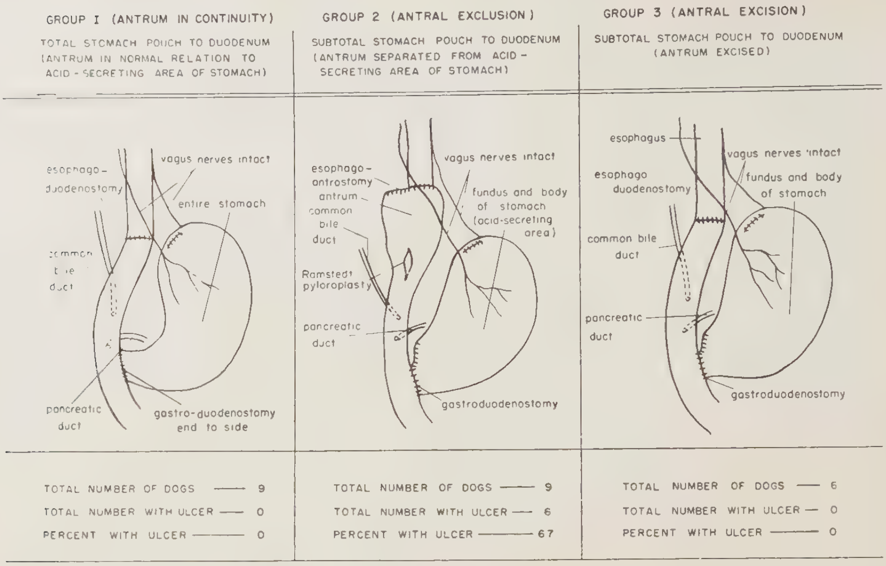
GROUP I (ANTRUM IN CONTINUITY)
TOTAL STOMACH POUCH TO DUODENUM (ANTRUM IN NORMAL RELATION TO ACID-SECRETING AREA OF STOMACH)
GROUP 2 (ANTRAL EXCLUSION)
SUBTOTAL STOMACH POUCH TO DUODENUM (ANTRUM SEPARATED FROM ACID-SECRETING AREA OF STOMACH)
GROUP 3 (ANTRAL EXCISION)
SUBTOTAL STOMACH POUCH TO DUODENUM (ANTRUM EXCISED)
esophago-duodenostomy
vagus nerves intact
entire stomach
common bile duct
pancreatic duct
gastro-duodenostomy end to side
esophago-antrostomy
antrum
common bile duct
Ramstedt pyloroplasty
pancreatic duct
vagus nerves intact
fundus and body of stomach (acid-secreting area)
gastroduodenostomy
esophagus
esophago duodenostomy
common bile duct
pancreatic duct
vagus nerves intact
fundus and body of stomach
gastroduodenostomy
TOTAL NUMBER OF DOGS — 9
TOTAL NUMBER WITH ULCER — 0
PERCENT WITH ULCER — 0
TOTAL NUMBER OF DOGS — 9
TOTAL NUMBER WITH ULCER — 6
PERCENT WITH ULCER — 67
TOTAL NUMBER OF DOGS — 6
TOTAL NUMBER WITH ULCER — 0
PERCENT WITH ULCER — 0

FIG. 1.

age involved operations in which the antrum was either excluded from contact with the acid-secreting segment of the stomach or completely excised. In none of his experiments was the stomach left intact.

The experiments reported here were devised to determine the effect of changes in the position of the antrum (relative to the parietal cell portion of the stomach) on gastric secretion of hydrochloric acid and on ulcer genesis. It was our thesis that the separation of the antrum from the remainder of the stomach caused the marked ulcerogenic effect of Sauvage's operation.

Methods. Adult mongrel dogs were anesthetised with pentobarbital sodium, 30 mg per kg of body weight. Intermittent positive pressure artificial respiration with compressed air was used. An incision was made in the tenth left intercostal space and the stomach and duodenum were exposed by opening the esophageal hiatus and drawing them into the chest. The esophagus was divided just above the esophagogastric junction, care being given to leave the vagus nerve trunks intact. The distal end of the divided esophagus was inverted with interrupted silk, leaving the stomach as a blind pouch. An end-to-end anastomosis was then accomplished between the proximal esophagus and either the duodenum or the gastric antrum. The blind vagally-innervated stomach pouch was then anastomosed end to side to the duodenum just below the papilla of the pancreatic duct. The esophageal hiatus was closed by suturing the esophageal curura of the diaphragm to the esophagus. In those dogs where the antrum was left in normal continuity with the duodenum, either a Ramstedt pyloromyotomy or a Heinecke-Mikulicz pyloroplasty was done to prevent any obstruction from pylorospasm which might attend division of the stomach with its consequent division of the vagal innervation of the pylorus. The study involved 3 different groups of dogs. In the dogs in Group I (Antrum in Continuity) the total gastric pouch was anastomosed to the duodenum end-to-side. In the dogs in Group II (Antral Exclusion) the antrum was divided from the parietal cell portion of the stomach. The proximal parietal cell pouch was anastomosed to the side of the duodenum, and intestinal continuity was established by anastomosing the esophagus to the antrum which retained its normal attachment to the duodenum. In the dogs in Group III (Antral Excision) the antrum was excised and the parietal cell pouch was anastomosed to the duodenum end-to-side. Three additional dogs having the Group I operation (Total Gastric

TABLE I. Heidenhain Pouch Data on Gastric Secretion in B Dogs in Groups I and II. (Values are milliequivalents of hydrochloric acid secreted during 8 hr collection period.)

Group I—Operation (total gastric pouch with antrum in continuity)

	Dog #C-1		Dog #C-2		Dog #C-3	
Stand	Pre Op	Post Op	Pre Op	Post Op	Pre Op	Post Op
1	7.86 mEq	1.00 mEq	1.04 mEq	.06 mEq	2.01 mEq	1.38 mEq
2	5.15	2.03	1.22	.19	.55	.14
3	6.34	.71	2.83	.19	.34	3.22
4	13.52	1.13	4.34	.45	2.22	1.93
5	9.01	1.17	1.92	1.61	1.82	1.46
6	16.32	2.63	2.56	.45	1.85	.98
7	11.07	1.01	1.33	.0	1.52	.65
8	9.09	.44	3.50	.14	1.50	1.73
9	9.69	1.76	3.97	.0	1.89	.46
10	2.81	1.95	3.01	.36	.67	
11	2.16	1.71	5.86	.44		
12	6.45	1.51	3.67	.0		
13		.42				
14		.65				
15		3.64				
Avg	8.29 mEq	1.45 mEq	2.94 mEq	0.32 mEq	1.37 mEq	1.33 mEq
Change	Down 82%		Down 89%		Down 3%	

Group II—Operation (antral exclusion)

	Dog #E-1		Dog #E-2		Dog #E-3	
Stand	Pre Op	Post Op	Pre Op	Post Op	Pre Op	Post Op
1	.48 mEq	1.28 mEq	1.26 mEq	2.77 mEq	1.59 mEq	13.23 mEq
2	.63	3.06	1.68	2.89	2.92	19.71
3	.67	5.06	2.35	6.30	2.30	17.45
4	1.26	2.16	1.51	1.48	.65	*
5	.69	1.89	.99	5.14	.18	
6	.58	.23	2.21	4.41	1.74	
7	4.38	2.16	3.22	1.84	.49	
8	1.98	7.37	.76	2.93	2.66	
9	2.14	8.38	1.08	3.86	3.41	
10	1.03	5.03	1.82		4.37	
11	1.51	4.00	.89		2.67	
12		3.65	.37		3.16	
13		9.74	.39		1.77	
Avg	1.40 mEq	4.15 mEq	1.43 mEq	3.51 mEq	2.15 mEq	16.80 mEq
Change	Up 197%		Up 146%		Up 681%	

* Dog died with chronic stomal ulcer on 23rd postop day.

Pouch with the Antrum in Continuity) and 3 additional dogs having the Group II operation (Antral Exclusion) had previously had isolated gastric pouches (Heidenhain pouches) made and standardized. Standard pouch collections were made over an 8-hour period 3 times weekly. The dogs were fasted for 16 hours prior to each collection. At the beginning of the 3rd hour of each collection, they were given a standard 200 g cooked horsemeat meal. Specimens were collected hourly and measured for free and total acid and volume. The values reported are the total milliequivalents of free hydrochloric acid secreted during each 8-hour collection period.

Results. The results of the survival experiments are tabulated in Fig. 1 and in Table I.

The 9 dogs in Group I (Antrum in Continuity) survived an average of more than 6 months which was the termination time for the experiment. None of these dogs developed an ulcer, and weight loss following this operation was minimal, the average being 10%.

Similarly, the 6 dogs in Group III (Antral Excision) lived an average of more than 6 months, and none developed an ulcer. The average weight loss in this group too was 10%.

On the other hand, 6 of the 9 dogs in Group II (Antral Exclusion) died with chronic peptic ulcers. The average survival of the 6 dogs with ulcer was 104 days, the range being 49 to 174 days. The weight loss in this group was severe—the average weight loss in the 6 dogs with ulcer being 36%. There was little or no difference in either survival time or ulcer development between those dogs with Ramstedt and those with Heinecke-Mikulicz pyloroplasties.

Most of the dogs in Group I and Group III had a moderately severe chronic esophagitis characterized by hyperemia of the mucosa, marked thickening of the esophageal wall, and moderate dilatation of the esophagus when they were sacrificed. All of the dogs in Group II in which the antrum, retaining its normal attachment to the duodenum, was anastomosed to the esophagus had normal esophagi when they died.

Results of the Heidenhain pouch observations are summarized in Table I.

Following the Group II operation (Antral Exclusion) hydrochloric acid secretion increased markedly. In 2 dogs, the average preoperative 8-hour hydrochloric acid secretion was 1.41 milliequivalents (average of 24 collections). Postoperatively, the average secretion rose to 3.89 milliequivalents (average of 22 collections), an increase of 175%.

On the other hand, following the Group I operation (Antrum in Continuity), the secretion from the Heidenhain Pouch decreased. Preoperatively, the average 8-hour hydrochloric output in 34 collections from 3 dogs was 4.37 milliequivalents, and postoperatively the average of 36 collections from the same dogs was 1.04 milliequivalents—a decrease of 76%.

Discussion. These experiments show the importance of the positioning of the antrum in experimental ulcer genesis. Only those animals with the gastric antrum excluded from the acid-secreting part of the stomach developed ulcers. No ulcers occurred in those dogs in which the antrum remained in continuity with the rest of the stomach, (Group I), nor in those dogs in which the antrum was excised (Group III).

The Heidenhain pouch data clearly explain the difference in ulcer incidence following these operations. A 175% increase in hydrochloric acid secretion followed the Group II operation (Antral Exclusion); a decrease of 76% in secretion followed the Group I operation (Total Gastric Pouch with the Antrum in Continuity).

The increase in gastric secretion in dogs with the antral exclusion operation (Group II) can be explained on the basis of increased production of gastrin by the antrum which is in prolonged contact with food and alkaline secretions such as saliva, and refluxed bile and pancreatic juice. The decrease in gastric secretion attending performance of the Group I operation (Total Gastric Pouch with the Antrum in Continuity) can be explained by the suppression of gastrin formation occasioned by the continuous flow of acid gastric secretion over the antral mucosa. In addition to this factor, there is a lesser degree of contact with food in the antrum in this operation, since the entire gastric pouch was not in the stream of flow of the intestinal contents; undoubtedly however, there is considerable reflux of food and intestinal content into the blind gastric pouch.

These experiments lend support for those operations(5,6) recently introduced in the treatment of peptic ulcer, in which a subtotal resection of the parietal cell segment of the stomach is done and the antrum is anastomosed to the residual parietal cell segment. For apparently the antral phase of gastric secretion is not nearly so great when the antrum retains its normal relation to the remainder of the stomach as when the antrum is excluded from contact with acid gastric juice.

Conclusions. 1. Exclusion of the antrum from continuity with the residual gastric pouch is the chief ulcerogenic factor in the Sauvage operation in which the antrum is anastomosed to the esophagus (Group II dogs). 2. Stomal peptic ulcer was not observed in vagally innervated total gastric pouches when the antrum remained in contact with the residual gastric pouch (Group I dogs). 3. Augmentation of the secretion of acid from an isolated Heidenhain pouch attends antral migration or exclusion (Group II dogs). Diminution of secretion from such a pouch was observed when the vagally innervated entire stomach, with the antrum in its normal position, was attached to the side of the duodenum as a blind pouch (Group I dogs).

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Propagation in Tissue Cultures of Cytopathogenic Agents from Patients with Measles.*† (21073)

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Numerous attempts have been made in the past to propagate the agent of measles in lower animals, in chick embryos and in tissue cultures(1-3). The results of different investigators were often at variance or directly contradictory. It has been made reasonably clear, however, that monkeys, especially *M. mulatta*, are moderately susceptible to experimental inoculation(3). Furthermore the researches of Rake, Shaffer and their collaborators have provided evidence suggesting that the agent which passed through bacteria-retaining filters could be maintained indefinitely in serial passages in the developing chick embryo(4,5). These workers(5) also confirmed the earlier observations of Plotz(6) who apparently had succeeded in growing the agent in a modified suspended cell culture of chick embryonic tissues. Egg passage in the hands of Shaffer and his coworkers(7,8) regularly appeared to alter the pathogenicity of the agent for man as indicated by the development of a mild and much modified disease following the inoculation of egg adapted materials into susceptible children. In certain cases this modified disease seemed to be followed by resistance to measles as indicated by the results of subsequent natural or artificial exposure to the virulent form of the agent(9). Since 1943 when the last of the communications by Rake and his collaborators appeared, no important progress has been made in the study of the etiology of measles. This fact may in large part be attributed to the lack of a convenient laboratory method for the demonstration of the presence of the agent which induced no recognizable changes in eggs or cultures of chick tissues. Moreover, repeated attempts by Shaffer(10) to demonstrate a serologic reaction, such as complement fixation, using materials from the infected chick embryo failed. Accordingly, the only available technics have consisted in the inoculation of man or the monkey. The former is obviously impractical as routine and the latter tedious, expensive and frequently inconclusive because of variation in individual susceptibility.

With these considerations in mind we have recently attempted to cultivate the agent of measles in cultures of human and monkey cells employing procedures applied successfully to the propagation of the poliomyelitis viruses(11-13). In blood and throat washings of typical cases of measles agents have been demonstrated that can be maintained in serial

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TABLE I. Cytopathogenic Agents Isolated in Tissue Cultures from Throat Washings and Blood of 5 Measles Cases.

Material and No. of passages				Time of collection of materials	
T.W.*	Passages	Blood	Passages	Days after onset	Hours after rash
—†	—	+	3	5	29
+	3	+	3	3	17
+	4	+	10	2	14
+	3	ND‡	ND	4	6
+	2	+	1	?	±24

* T.W. = Throat washings.
† No virus isolated.
‡ ND = Not done.

passage in tissue cultures and which induce distinctive cytopathic changes in renal epithelial cells. A certain amount of evidence has been accumulated indicating that antibodies specific for these agents develop during the course of the disease. It is our purpose to describe here these observations in a preliminary manner. Additional evidence for the relationship of these agents to measles will be sought in future investigations.

Materials and methods. Collection of specimens. Throat washings, venous blood and feces were obtained from 7 patients as early as possible after a clinical diagnosis of measles was established. In 5 instances the time at which specimens were collected in relation to the onset of exanthem is given in the case histories described below or in Table I. When capable, patients were asked to gargle with 10-15 ml of sterile neutralized fat-free milk. Certain specimens from the throats of younger children were obtained by cotton swab previously moistened in milk. After swabbing the throat the swab was immersed in 2 ml of milk. Penicillin, 100 u/ml, and streptomycin, 50 mg/ml, were added to all throat specimens which were then centrifuged at 5450 rpm for about one hour. Supernatant fluid and sediment resuspended in a small volume of milk were used as separate inocula in different experiments in amounts varying from 0.5 ml to 3.0 ml. About 10 ml of blood immediately after withdrawal were placed in tubes containing 2 ml of 0.05% solution of heparin. As inocula for tissue cultures amounts varying from 0.5 ml to 2.0 ml of the whole blood were employed. After addition of antibiotics as described above 10% fecal suspensions were prepared by grinding the material in bovine amniotic fluid medium. The suspensions were then centrifuged at 5450 rpm for about one hour and the supernatant fluids used as inocula, in amounts varying from 0.1 ml to 3 ml. All specimens were refrigerated in water and ice or maintained in the cold at about 5°C from the time of collection until they were added to the cultures. The maximum time that lapsed between collection of specimens and inoculation was 3½ hours.

Tissue culture technics. In the initial isolation attempts roller tube cultures(11,12) of human kidney, human embryonic lung, human embryonic intestine, human uterus and rhesus monkey testis were employed. Subsequent passages of the agents isolated were later attempted in human kidney, human embryonic skin and muscle, human foreskin, human uterus, rhesus monkey kidney and embryonic chick tissue. Stationary cultures prepared according to the technic of Youngner(13) with trypsinized human and rhesus monkey kidney were later employed for isolation of agents and their passage. The culture medium consisted of bovine amniotic fluid (90%), beef embryo extract (5%), horse serum (5%), antibiotics, and phenol red as an indicator of cell metabolism(12). Soybean trypsin inhibitor was added to this medium unless it was used for the cultivation of human and monkey kidney (11). Fluids were usually changed at intervals of 4-5 days. For histological examination the cell growth after fixation in 10% formalin was embedded in collodion, dehydrated and stained with hematoxylin and eosin.‡

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‡ We are indebted to Dr. William J. Cheatham for the following account of the collodion embedding technic and for the preparation of material for histologic examination. *Collodion membrane technic.* The culture fluid is removed from the roller tube and replaced by fixative sufficient to cover the areas of outgrowth; formalin has generally been used. Fixation for one hour is adequate with the method of tissue culture used. Following fixation, dehydration is carried out with increasing concentrations of alcohol ending in ether-alcohol mixture (50 per cent of each).

Manner of passage in tissue culture. Serial passage of the various strains (Table I) was accomplished as routine by removal of the culture medium between the 4th and the 16th day after inoculation and immediate transfer of 0.1 ml to each of a number of fresh cultures. Successful passage of the agent with fluids that had been previously centrifuged at 2500 rpm to remove cellular elements has also been repeatedly demonstrated. Larger inocula (up to 1.0 ml) were often used during the initial experiments before the resistance of the agent to storage at various temperatures had been determined. *Virus neutralization and complement fixation tests.* The procedures employed are described subsequently in the text. *Description of cases from which materials were obtained.* During an outbreak of measles at a private boarding school for boys in Southboro, Mass., isolations were attempted from throat washings and stools of 4 patients and blood of 3 of the same patients. The latter are designated as Cases 1, 2 and 3. Throat washings of 2 siblings in a small epidemic of measles in Wellesley, Mass., (Cases 4 and 5) and blood from 2 cases of measles admitted to the Boston City Hospital during an epidemic in Boston (Cases 6 and 7) are under investigation at the present time. Details of these typical cases are omitted for the sake of brevity.

Case 1: D.R., age 11, was in contact with a "probable" case of measles 10 days before symptoms commenced on 1/20/54. The latter consisted of signs of an upper respiratory infection including sore throat and fever to 101°F. He soon developed conjunctivitis and a bad cough. These symptoms became aggravated and on 1/24 in the morning there was a suggestion of a rash on his face. Temperature at 4:30 p.m. was 105.5°F. Koplik's spots were noted on the buccal mucosa by the school physician the next morning. On 1/25 at 1 p.m. he was seen by TCP with findings of temperature 98° p.o., mild to moderate conjunctivitis, moderate generalized adenopathy and characteristic blotchy maculopapular rash in full bloom, extending to involve even the palms and soles. No Koplik's spots were seen at this time. Specimens of throat washings, blood and stool were collected at 1:30 p.m. on 1/25. *Case 2:* H.J., age 13, with no known contact other than Case 1, developed signs of an upper respiratory infection on 1/26/54. He complained of sore throat and cough the following day and of photophobia on 1/28. At this time the infirmary nurse noticed a questionable trace of a rash on his forehead. He was seen on 1/29 by TCP with findings of temperature 101° p.o., and maculopapular rash developing over the face, mildly over the chest and abdomen, minimally on the upper extremities and none on the lower extremities. He had moderate conjunctivitis, cough and Koplik's spots on the buccal mucosa. Specimens of throat washings, blood and stool were obtained at 11 a.m. on 1/29. *Case 3:* D.E., age 13, a close contact of both Cases 1 and 2 during their prodromal stages, experienced a gastrointestinal upset on 2/8/54 with cramps and nausea. Temperature was 99.6°F. Faint rash was first noted on his face in the late evening of 2/9, and he was seen by TCP at 12 noon on 2/10 with findings of typical rash over the face with minimal extension to the chest, abdomen and back. Koplik's spots

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The latter is then replaced by a dilute solution of collodion in ether-alcohol allowing an hour or more for penetration of the collodion into the tissues. The concentration of collodion is best determined by trials in which films are removed from blank tubes. The collodion is poured from the tube, rotating the latter so that the entire inner surface is covered by a thin film. The collodion is allowed to dry partially, rotating the tube constantly. This requires 1-2 minutes and can be arrived at by experience, employing as a guide the time at which the odor of ether-alcohol is barely detectable. Cold water is then run slowly into the tube to harden the membrane. The upper edge of the tube is rimmed and the membrane carefully separated from the glass with a thin glass rod. With patience a cast of the tube including the tissue is obtained; this is cut into a flat sheet which can be stained and trimmed according to the distribution of tissue. The technic of staining with hematoxylin and eosin is used and need not be described in detail here. Alcoholic eosin has been found most satisfactory. Complete dehydration using absolute alcohol cannot be used because of the solubility of collodion. Clearing is therefore carried out from 95 per cent alcohol in oil of Origanum followed by xylol. After clearing the membrane is mounted using any satisfactory mounting media.

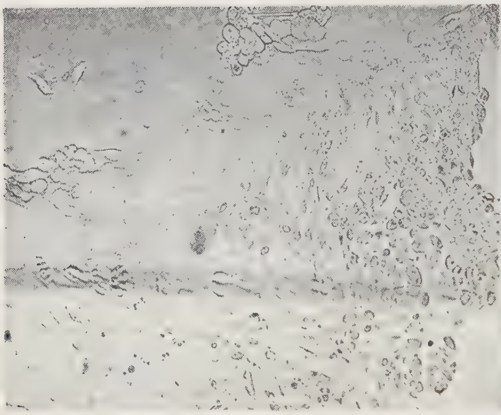

Fig. 1.

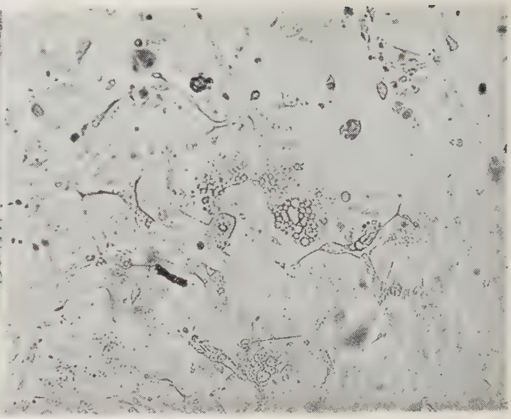

Fig. 2.

FIG. 1. Outgrowth of normal human kidney cells in an uninoculated roller tube culture (×130). Control for cultures shown in Fig. 2 and 3.

FIG. 2. Area of syncytial giant cells with small cytoplasmic vacuoles. Note many nucleoli and faint nuclear outlines. 9th day after inoculation; 7th passage, agent from Case 3 blood (×130).

were present on the buccal mucosa. Cough and conjunctivitis were mild. His temperature was 102° p.o. Specimens of throat washings, blood and stool were obtained at this time. His temperature was 103.6°F with rash in full bloom in the afternoon. On the following morning his temperature was 104°F.

Experimental. Cytopathic changes induced by agents isolated from cases of measles. The first of 8 agents obtained from blood or throat washings of measles cases and exhibiting comparable properties was isolated in cultures of human kidney tissue following addition of the blood of Case 3. In each of the 3 cultures that were inoculated cytopathic changes were observed on the 7th day. Since these changes presented a characteristic appearance not heretofore associated definitely with a virus they have provided the means for the further investigation of this agent as well as others that have been recently isolated. Accordingly, here at the beginning these changes will be described in detail. Observation of *fresh preparations* under low magnification (80X) revealed within the sheet-like outgrowth of renal epithelial cells discrete areas of varying size and shape in which the cell boundaries were obliterated and the nuclei often difficult to visualize. Within these areas, which may be described as non-refractile "glassy" plaques, large and small vacuoles were often numerous lending them a foamy or lace-like quality. The number and size of the vacuoles increased as incubation was continued. On careful examination of these areas many small, slightly refractile bodies were seen that resembled nucleoli within nuclei whose outlines could often be distinguished only with difficulty. The total effect thus suggested the presence of large vacuolated giant cells. After further cultivation the extent of the areas initially present was slowly extended or was enlarged by coalescence with neighboring plaques while others developed elsewhere. In addition to the formation of vacuoles degenerative changes gradually appeared within the affected areas suggesting coagulation necrosis. At the end of three weeks most of the epithelial cells appeared to be involved, yet here and there small aggregates of normal cells remained. These seemed, however, to be composed mainly of spindle-shaped cells. Reference to Fig. 1, 2 and 3 will aid in the visualization of these changes as they are manifest in the natural state. In contrast to the appearance of the normal cell outgrowth shown in Fig. 1 the smooth confluent area of affected cells stands out clearly in Fig. 2. While a slight degree of vacuolization is evident in this figure, it is extensive in Fig. 3 especially along the margin of cell growth where it is first apt to become apparent.

The interpretation that has just been presented of the changes observed in fresh prepa-

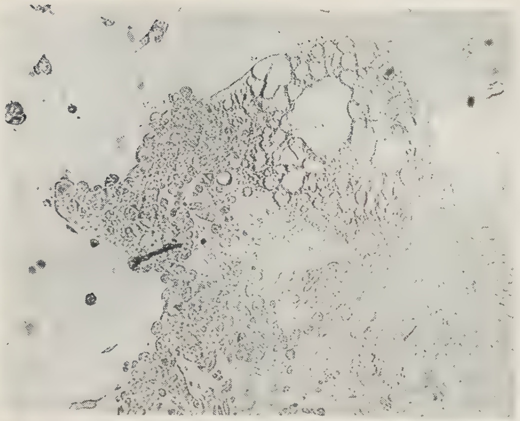

FIG. 3. Lace-like network of vacuoles at tissue margin from the same culture (×130).

rations was supported by examination of fixed and stained materials. Under these conditions the glassy areas were clearly revealed as collections of nuclei surrounded by a common cytoplasmic matrix. As many as 40 to 100 nuclei were counted in such syncytial formations. Often the limits of the encompassing cytoplasm were sharply defined thus contributing to the impression that development of true giant cells has occurred *in vitro*. Whether or not this is actually the case, the phenomenon is of much interest in view of the constant presence of giant cells in lymphoid tissues during the early stages of measles in man(14,15).

Examination of *stained materials* also revealed significant changes within the nuclei of the giant cells that were not visible in fresh preparations. These consisted in a redistribution of the chromatin which ultimately assumed a marginal position where it formed a dense ring or crescent that stained intensely with the basic dye. Concomitantly the central portion of the nucleus came to be occupied by an apparently homogeneous substance, acidophilic in character, that approximated closely to the chromatin ring. Since in these and other preparations that have been examined subsequently no clear unstained zone has been observed between the chromatin and this acidophilic mass, it cannot be asserted that the latter represents an intranuclear inclusion body of the type characteristically associated with viral infections. Nevertheless, as far as can now be determined, its presence along with the margination of the chromatin affords a useful criterion of infection for the agents under study. It should be emphasized, however, that the changes as just depicted are encountered in cultures that have been incubated for relatively prolonged periods (*e.g.* 14-21 days). When the interval between inoculation of the agent and examination of the stained cells (*e.g.* 4 days) is shorter, margination of the chromatin may be incomplete or inapparent and the acidophilic substance may only be seen in small rounded masses distributed here and there amid nuclear materials that approximate the normal arrangement. Fig. 4, 5, 6 and 7 illustrate well-developed nuclear changes and also the general similarity of the affected areas to the giant cells encountered in lesions associated with measles. Of particular interest in this latter connection are the basophilic "pseudoprotozoal" bodies that Bonenfant(16) has recently described in the mucosa and lymphoid follicles in cases of this disease. These bodies were usually surrounded by an acidophilic homogeneous substance. As described and pictured these bodies with their matrix strikingly resemble the giant cells in tissue cultures that exhibit well-developed nuclear changes.§

Some biologic properties of the agents isolated from measles. Certain of the biologic properties of the agents isolated from patients with measles have been definitely determined, others in a preliminary or tentative manner. In several instances these properties have been studied only in respect to the strain first isolated from the blood of Case 3. Since, however, the other strains, as far as they have been examined, behave in a similar manner it is probable that all of them, when thoroughly studied, will exhibit the same general characteristics.

A) *Source of virus.* As repeatedly stated, agents have been recovered from both blood and throat washings. In three cases yielding viruses from one or another of these sources

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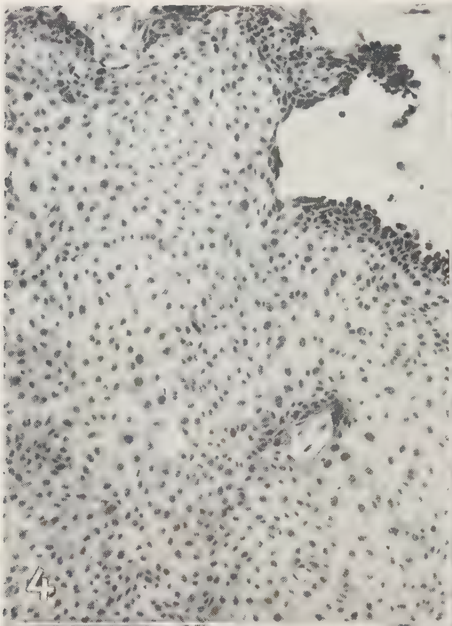
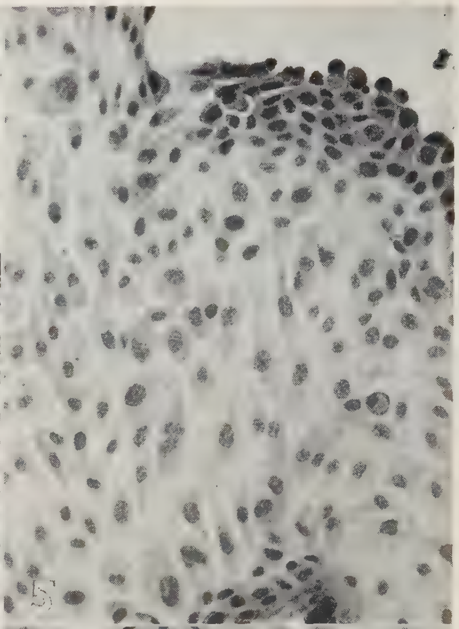
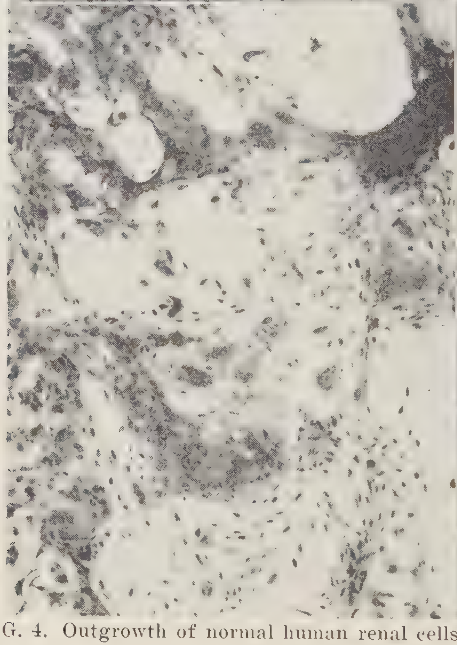
§ Since this manuscript was submitted for publication materials fixed in Bouin's fluid, employing the usual alteration of washing with 70 per cent alcohol following fixation, have been examined. Typical acidophilic intranuclear inclusion bodies were regularly seen surrounded by a clear zone or "halo."







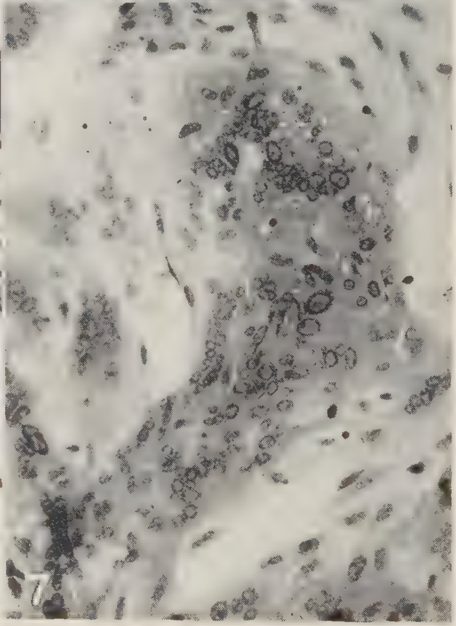


FIG. 4. Outgrowth of normal human renal cells fixed and stained with hematoxylin and eosin. Control for cultures shown in Fig. 5-7 (×110).

FIG. 5. A portion of the cell outgrowth shown in Fig. 4 more highly magnified. Hematoxylin and eosin stain (×300).

FIG. 6. Outgrowth of human renal cells showing giant cell formation and nuclear changes 20 days after inoculation with 2 ml blood from Case 3. Hematoxylin and eosin stain (×110).

FIG. 7. A portion of cell outgrowth shown in Fig. 6 more highly magnified. Hematoxylin and eosin stain (×300).

fecal suspensions have likewise been examined by the tissue culture technic. In none was evidence for the presence of an agent obtained. Further examinations of fecal specimens are necessary before it can be stated whether or not the virus is present in the intestinal excreta.

B) *Cytopathogenic range.* Monkey kidney is the only other tissue employed that has yielded a growth of cells in which the characteristic changes described above have been definitely observed following inoculation of virus. In cultures consisting largely of monkey renal epithelial cells as prepared by Youngner's modification of Dulbecco's technic (13) cytopathic changes have been regularly observed which resemble closely those produced by these agents in human renal cells as seen in both fresh and stained preparations. These effects followed the addition of blood or throat washings from cases of measles as well as infected tissue culture fluids derived from previous passages. Monkey kidney cultures may, therefore, be applied to the study of these agents in the same manner as cultures of human kidney. In so doing, however, it must be borne in mind that cytopathic effects which superficially resemble those resulting from infection by the measles agents may possibly be induced by other viral agents present in the monkey kidney tissue (*cf.* last paragraph under G) or by unknown factors. In a few cultures of human prepucial tissue inoculated with one of the measles agents changes resembling those seen in renal cells were noted in the epithelial outgrowth about certain fragments. Additional observations, however, will be required before it can be confidently asserted that dermal epithelial cells are specifically attacked by these viruses. In a single experiment no cytopathic manifestations were seen during a period of 31 days following inoculation of infected tissue culture fluid into cultures of human embryonic skin and muscle, human uterine tissue or embryonic chick tissue. Tests for the presence of complement fixing antigen in the fluids removed from the cultures on the 31st day were negative. These serologic results suggest that growth of the virus did not occur, since, as will be shown subsequently, the antigen appears to develop regularly after several days in cultures of renal tissue infected with the virus.

C) *Failure to induce demonstrable changes in mice or chick embryos.* Two litters of suckling white mice (1-day-old) were inoculated with infective tissue culture fluid by both the intraperitoneal and intracerebral routes. The animals remained well during an observation period of 21 days. Employing the same material as inoculum 0.1 ml amounts were introduced into the amniotic sac of 7-day embryonated hen's eggs. After 7 days incubation at 36°C the amniotic fluid and membranes were harvested, ground with alundum and centrifuged at 1500 rpm. The supernatant fluid was used for a second egg passage which was carried out in the same manner. Whereas inoculation of 0.1 ml aliquots of the first egg passage material into cultures of monkey renal epithelium was followed by characteristic cytopathic changes on the 8th day, the addition of 0.5 ml of second egg passage material to such cultures failed to produce this effect. No complement fixing antigen was detected in the materials from the egg passages. Although these results suggest that the virus is not readily adapted to growth in the chick embryo, it is evident that much further investigation will be required to determine the degree of susceptibility of this host.

D) *Serial passages.* Serial passages of several of the strains have been carried out without difficulty in cultures of human or monkey renal cells. Employing 0.1 ml of the fluid phase as inoculum, 10 passages of the first strain isolated have so far been accomplished in human cells of this type. With other strains fewer passages have been completed as indicated in Table I.

E) *Assay of infectivity.* As yet only one attempt has been made to measure infectivity of virus propagated in tissue culture. In this case fluids and cells were removed from 15 cultures of human kidney tissue on the 6th day after inoculation of fluid from the 4th tissue culture passage of the agent from Case 3. These materials were pooled and the cells were ground with alundum in the presence of the fluid. After centrifugation for 15 minutes at 2500 rpm the supernatant fluid was titrated for infectivity in cultures of human kidney tissues. For this purpose 3 cultures were each inoculated with 0.1 ml of the suspension diluted by a factor of 10. The endpoint of viral activity as indicated by the highest dilution causing cytopathic changes was about $10^{-2.5}$. This low titer was somewhat unexpected in view of the fact, as will be shown here-

TABLE II. Neutralization of Cytopathogenic Effect of Virus from Measles Case 3 by Convalescent Sera from Two Other Cases of Measles.

Serum source	Serum drawn (days after rash)	Neutralizing serum dilution*
Case 1	1	<1:8
	17	1:32
2	0	<1:2
	12	1:32
	70	1:32

* Readings taken on 10th day after addition of virus.

after, that tissue culture fluids contain sufficient antigen to fix complement in the presence of convalescent measles serum. Without more experimental data, however, it cannot be assumed that maximal infectivity titers lie within this range.

F) *Neutralization of cytopathogenicity by convalescent measles sera.* That the cytopathogenic capacity of at least one strain of the agents associated with measles is inhibited by serum factors developing during the course of the disease has been demonstrated in two experiments. Employing 100 ID_{50} of the viral suspension mentioned in the previous paragraph neutralization tests were carried out in cultures of monkey renal epithelial cells. Sera taken during the acute and convalescent stages from two of the cases occurring at the boys' school were stored at –15°C and inactivated at 56°C for 30 minutes before they were diluted and used in the test. As diluent bovine amniotic fluid was employed. Dilutions of serum and virus were mixed and kept at 5°C for one hour when 0.1 ml of each mixture was added to each of three tissue cultures. In both tests the cultures were examined every day or every 2 days and the final readings were recorded on the 10th day. The results are summarized in Table II. They indicate that significant increases in substances occurred in the serum of both patients that neutralized the cytopathogenicity of the agent isolated from the blood of a third patient. In considering these results it is pertinent to recall that agents with similar characteristics have been isolated from the two patients whose convalescent sera were shown to possess virus-neutralizing capacity.

G) *Production of complement fixing antigen in tissue cultures.* Since it has been shown (17) that in cultures of poliomyelitis viruses antigens capable of fixing complement in the presence of specific antibodies appear in the fluid phase, tests were carried out to determine whether or not the fluids removed from cultures exhibiting cytopathic changes induced by the measles agents might behave in a similar manner. To this end the drop method of Fulton and Dumbell(18) as modified by Svedmyr, Enders and Holloway(17) was employed. As antigens crude undiluted fluids have been used. These were derived from human or monkey kidney cultures inoculated with strains isolated from either blood or throat washings of Cases 2, 3 and 4. The fluids from several cultures were collected at various intervals after inoculation of the virus, pooled, centrifuged at 1500 rpm for 5 minutes and stored at either 5°C or –16°C. Immediately before use the fluid was heated at 56°C for 30 minutes to remove any anticomplementary activity that might be present. As control antigens, fluids were taken from uninoculated cultures maintained under the same conditions as well as fluids from cultures of the agent producing changes superficially similar to those caused by the measles agents and which are mentioned below. These materials failed to fix complement with any of the sera that have been examined. Acute and convalescent phase measles sera were inactivated at 56°C and serial dilutions prepared as in complement fixation tests for poliomyelitis antibody. The results of tests that have so far been completed indicate clearly that antibodies develop during the course of measles capable of reacting with an antigen that appears in the culture fluid after the 3rd to the 7th day following inoculation of the virus. From representative data presented in Table III it is evident that the antibody may emerge at least as early as the 7th day following the appearance of the rash and continues to persist for at least 2 months in fairly high titer. By this time, however, there is some indication that the maximal concentration has been previously attained. It is noteworthy in respect to the possible etiologic relationship of these agents to measles that antibodies appeared in the blood of Cases 6

TABLE III. Results of Complement Fixation Tests on Measles Sera Employing as Antigens Fluids from Tissue Cultures Infected with Agents Isolated from Cases of Measles.

Serum source (case No.)	Serum drawn (days after rash)	CF titer (serum dilution) Antigen MKC*	Antigen HKC†
1	1	<1:8	1:8
	17	1:256	1:512
	75	1:64	1:128
2	0	<1:8	<1:8
	12	1:128	1:256
	70	1:64	1:128
3	0	<1:8	<1:8
	58	1:256	1:256
6	0	<1:8	<1:8
	7	1:32	1:32
7	1	<1:8	<1:8
	8	1:64	1:128

* MKC = Monkey kidney tissue culture fluid infected with agent from blood of case 2.

† HKC = Human kidney tissue culture fluid infected with agent from blood of case 3.

and 7 which fixed complement with the antigens from Cases 2 and 3. The latter occurred in a widely separated area and at an earlier time. A few tests have been carried out with measles antigen on sera from 3 adults giving a history of measles during childhood. Serum titers of 1:2, 1:8 and >1:16 were recorded.

Filterability and resistance to physical agents. Filtration. A portion of the pool of virus with infectivity titer of $10^{-2.5}$ (*cf.* last paragraph under E) was diluted 1:5 in beef infusion broth and passed through a sintered glass filter under a pressure of 45 mm Hg. Time of filtration was 10 minutes. The capacity of the filter to retain *Serratia marcescens* was then demonstrated. The viral filtrate was shown to be free of bacteria by addition to infusion and thioglycollate broth and blood agar media. Five cultures of monkey renal cells were inoculated with the filtrate (0.5-1 ml). Characteristic cytopathic changes were subsequently noted in all. *Thermal stability.* The cytopathogenicity of one strain was destroyed by heating at 65° for 30 minutes. The infectivity of heparinized blood or throat washings in milk as tested in tissue culture was preserved for at least 3½ hours by refrigeration at 1°C to 5°C. Agents present in tissue culture fluids remained infective after 38 days at −15°C and after at least 35 days at about −50°C to −60°C.

Other agents isolated during this study. Two agents have been isolated while the present work was in progress that appear unrelated to those we have just described. The first was recovered from the throat washings of a typical case of measles occurring in the boys' school. Its wide cytopathogenic range, the character of the cytopathic changes induced and the fact that its infectivity for tissue cultures was neutralized by herpes simplex immune rabbit serum served to define its nature. A second agent was obtained from an uninoculated culture of monkey kidney cells. The cytopathic changes it induced in the unstained preparations could not be distinguished with confidence from the viruses isolated from measles. But, when the cells from infected cultures were fixed and stained, their effect could be easily distinguished since the internuclear changes typical of the measles agents were not observed. Moreover, as we have already indicated, fluids from cultures infected with the agent failed to fix complement in the presence of convalescent measles serum. Obviously the possibility of encountering such agents in studies with measles should be constantly kept in mind.

Discussion. Of the numerous experiments that have been reported in the past describing the successful isolation of the etiologic agent of measles only those in which monkeys were employed as the experimental animal have been consistently confirmed by other workers. Great caution should therefore be exercised in the interpretation of any new claims that the virus has been propagated in other hosts or systems. Accordingly, the results that are summarized here must be subjected to the most critical analysis.

The following facts tend to support the hypothesis that the viruses we have described are responsible for the disease. Experimentally transmissible agents exhibiting a similar and characteristic cytopathogenic effect in cultures of human or simian epithelial cells have been isolated from either the blood or throat washings derived from 5 of 7 typical cases of measles during the early acute phase. An agent was demonstrated in the blood of 4 of the 5 cases from which specimens were obtained and examined by the tissue culture method. These findings would seem to be of especial significance since it is unlikely that viruses unrelated to measles would be regularly present in the circulating blood of these individuals some of whom were geographically widely separated.

The pathologic changes induced by the agents in epithelial cells in tissue culture resemble, at least superficially, those found in certain tissues during the acute stage of measles. While there is no ground for concluding that the factors *in vivo* are the same as those which underlie the formation of giant cells and the nuclear disturbances *in vitro,* the appearance of these phenomena in cultured cells is consistent with the properties that *a priori* might be associated with the virus of measles.

The emergence of antibodies during the course of the disease capable of suppressing the cytopathogenic effect and of fixing complement in the presence of infected tissue culture fluids affords further evidence for the close association of the agents with measles. Obviously additional data to be derived from tests with sera from a large number of cases of measles as well as other infectious diseases, especially the common exanthemata, are desirable in order to eliminate any remaining doubt concerning the specificity of these serologic reactions. The accumulation of such data is now in progress.

Although we have thus already obtained considerable indirect evidence supporting the etiologic role of this group of agents in measles, 2 experiments essential in the establishment of this relationship remain to be carried out. These will consist in the production of measles in the monkey and in man with tissue culture materials after a number of passages *in vitro* sufficient to eliminate any virus introduced in the original inoculum. The recovery of the virus from the experimental disease in these hosts should then be accomplished.

Conclusion. The findings just summarized support the presumption that this group of agents is composed of representatives of the viral species responsible for measles.

Summary. Eight agents exhibiting the properties of viruses have been isolated in cultures of human or simian renal cells from the blood or throat washings of five cases of typical measles. Multiplication of the agents *in vitro* is accompanied by characteristic changes in the cells. Primarily these changes consist in the formation of syncytial giant cells wherein the chromatin assumes a marginal position and is replaced centrally by an acidophilic substance of unknown nature. The cytopathogenic effect of at least one of the agents is inhibited by convalescent phase measles sera from other patients with measles. Antigen appears during cultivation *in vitro* of the measles agents that reacts specifically in complement fixation tests with convalescent phase measles sera.

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Influence of Cortisone on Rate of Absorption and Formation of Liver Glycogen by Alanine.* (21074)

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Gluconeogenesis from protein in the fasting animal is believed to be mediated by the adrenal cortex(1-4). Hess and Shaffran(5) found that cortisone did not increase the production of liver glycogen after feeding glycine above the additive effect of the individual action of the 2 compounds. Glycine forms liver glycogen rather slowly, reaching a peak value at 14 hours(6) and it was possible that the slow rate of glycogen formation may have been responsible for the failure to demonstrate any effect by cortisone. Alanine has been reported to form liver glycogen more rapidly than glycine(7-9) and its rate of absorption has been found to range from 50 to 94 mg per 100 g per hour for DL alanine, 78 to 82 mg for L(+) alanine and 62 mg for D(—) alanine(8-12). The rate of absorption of glycine was somewhat faster, averaging 102 mg per 100 g per hour(6). Since alanine is more glycogenic than glycine and appears to be absorbed more slowly it was decided to repeat the study on the effect of cortisone on glycogen formation using alanine. Preliminary to this the rate of absorption of the several isomers of alanine and also the rate of liver glycogen formation were restudied.

Experimental. Determination of rates of absorption and glycogen formation. The same procedure was used as previously described (6), DL, D(—), or L(+)-alanine, dissolved in water, was administered at levels of 100, 150, 300, and 450 mg per 100 g body weight for 0.5-, 1-, 2-, and 3-hour periods, respectively. For the longer time periods 450 mg/100 g was used. Alanine was determined in the extracts of the gastrointestinal tract by the method of Alexander and Seligman(13), and liver glycogen by the method of Good, Kramer, and Somogyi(14). The absorption rates are corrected for 4.5% loss of alanine that occurred in the procedure. Absorption rates were not determined after the 3-hour period since the maximum dose given, 450 mg/100 g body weight, would have been completely absorbed. The glycogen values for periods of time longer than 6 hours are averages of determinations on from 4 to 6 animals.

TABLE I. Rate of Absorption of and Glycogen Formation by Alanine.

Isomer	No. of animals	Time, hr	Rate, mg/100 g/hr	Glycogen, %
DL	6	2	108	.30†
	5	3	110	.88
	4	4	112	2.00
	4	6		3.33
			Avg 109.0 ± 10.4*	
L (+)	4	2	109	.08
	4	3	112	.55
	4	6		3.50
			Avg 110.0 ± 4.3	
D (—)	5	2	82	.10
	4	3	92	1.00
	5	6		3.90
			Avg 85.3 ± 9.0	

* Stand. dev.

† Glycogen content of livers of 12 control rats averaged 0.04%.

DL-alanine was absorbed (Table I) at a faster rate than found by Lewis(10-12) but at approximately the same rate reported by Wingo and Smith(9). The rate of absorption of D(—) is somewhat less than that of L(+)-alanine, an observation also made by Lewis(10,11). Gibson and Wiseman(15), measuring the absorption of alanine from isolated loops of rat intestine and using about one tenth of the amount of alanine used in our experiments, found that the absorption rate of L(+)-alanine was twice that of D(—)-alanine. Alanine is more rapidly glyconeogenic than glycine, the peak with DL-alanine (Fig. 1) is reached at 6 hours and with glycine(6) at 14 hours. The rate of absorption of alanine is not greatly different from that of glycine, certainly not enough to explain the

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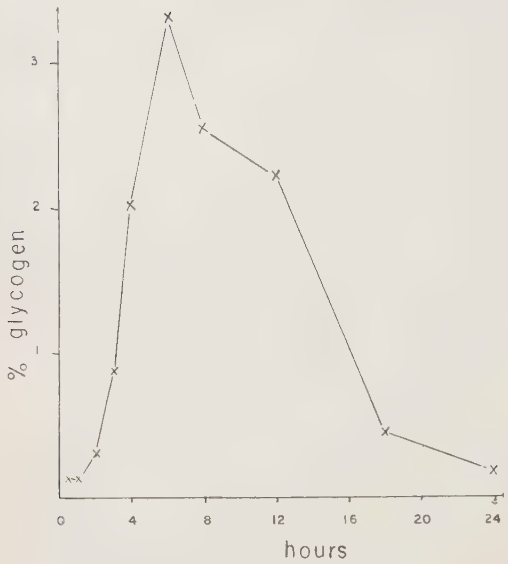

FIG. 1. Formation of liver glycogen by DL-alanine.

difference in the rate of liver glycogen formation. Reid(8) found a similar difference in rate and suggested that the metabolic pathways of the 2 amino acids are different. Both D(—)- and L(+)-alanine produced approximately the same amount of glycogen at the 6-hour period.

Administration of cortisone and alanine. The same procedure was employed as in the glycine experiments(5), 450 mg of DL-alanine dissolved in 2.5 ml H_2O were fed. Cortisone acetate in saline suspension (Cortone Merck) was given intramuscularly. It was found that cortisone in none of the dosage levels used had any effect on the absorption rates of the alanines tested. When alanine and either 2.5 or 5 mg of cortisone were given at the same time and the animals sacrificed after 6, 18, or 24 hours no significant increases in liver glycogen content above the sum of the amounts produced by DL-alanine and cortisone alone were found (Table II). This is the same result found when both glycine and cortisone were given together(5).

Since alanine forms glycogen at a faster rate and to a greater extent than glycine it is possible that by the time the maximum effect of the cortisone occurs the peak of glycogen production has been passed by several hours. Consequently an experiment was planned so that the peak effect of both the cortisone and the DL-alanine would occur at approximately the same time. Cortisone alone produced the maximum amount of glycogen after 24 hours and DL-alanine after 6 hours. By giving DL-alanine 18 hours after the cortisone the peaks for both compounds would coincide at 24 hours. In Table III the results of this experiment using 5 and 2.5 mg of cortisone are given and also the effect of feeding DL-alanine 1, 2, 4, and 6 hours before the cortisone peak. The values in column 3 are on the control animals given only cortisone and sacrificed after 24 hours. The values in column 4 are the differences between the values in column 1 and the sum of the alanine values for 1, 2, 4, and 6 hours given in column 2, taken from Fig. 1, and the values in column 3. Significant increases in glycogen content result when the peaks of cortisone activity and glycogen production from alanine are close together, particularly after 5 mg of cortisone. To ascertain whether a similar result could be obtained with glycine this amino acid was given so that its maximum period would occur at the same time as the cortisone maximum. The peak for glycine alone is at 14 hours therefore it was fed 10 hours after the cortisone was injected and the animals sacrificed at 24 hours, no extra liver glycogen was produced.

Total nitrogen determinations were made on the urines of the rats receiving 5.0 mg of cortisone for 24 hours and 450 mg of DL-alanine during the last 6 hours and also on 4

TABLE II. Liver Glycogen Formation by Simultaneous Administration of Cortisone and DL-Alanine.

Time, hr	1 Alanine + cortisone	2 Alanine	3 Cortisone (5)	4 Change
	%			
6 (4)*†	3.5	3.3	.1	+.1
18 (4)*	1.8	.5	.6	+.7
24 (6)*	1.7	.2	2.1	−.6
6 (4)‡	4.5	3.3	1.6	−.4
18 (4)‡	3.3	.5	2.2	+.6
24 (6)‡	2.5	.2	1.9	+.3

* 2.5 mg cortisone.
† No. in parentheses is No. of animals used.
‡ 5.0 mg cortisone.
Column 4 is the difference between column 1 and the sum of columns 2 and 3.

TABLE III. Cortisone Given 24 Hours, DL-Alanine Given Varying Number of Hours following Cortisone, and Subsequent Liver Glycogen Determinations.

Alanine, hr after cortisone	1 Alanine + cortisone	2 Alanine	3 Cortisone (5)	4 Change
	%			
23 (4)*†	2.7	.1	2.1	+ .5
22 (4)*	3.0	.3	”	+ .6
20 (4)*	4.9	2.0	”	+ .8
18 (6)*	6.6	3.3	”	+1.2
23 (4)‡	2.5	.1	1.9	+ .5
22 (4)‡	4.0	.3	”	+1.8
20 (6)‡	6.3	2.0	”	+2.4
18 (6)‡	7.3	3.3	”	+2.1

* 2.5 mg cortisone.

† No. in parentheses is No. of animals used.

‡ 5.0 mg cortisone.

Column 4 is the difference between column 1 and the sum of columns 2 and 3.

control rats receiving the same amount of DL-alanine for the last 6 hours of a 24-hour fasting period but with no cortisone. The average excretion of nitrogen on DL-alanine alone was 220 mg and for the cortisone plus alanine animals it was 250 mg. The nitrogen excretion of rats fasting for 24 hours was 190 mg and for rats receiving 5 mg of cortisone during the 24 hours fast the nitrogen excretion was 210 mg. There was a slight increase in nitrogen excretion from the DL-alanine when cortisone was administered, the extra nitrogen was equivalent to 40% of the alanine nitrogen when fed alone and 60% when cortisone was also administered.

Apparently there is a stimulation of glycogen formation by cortisone when alanine is fed but not with glycine. This may also point to a difference in the metabolic pathways of these 2 amino acids as suggested by Reid(8) and noted in connection with the rate of liver glycogen formation. It is planned to study this problem further by the use of isotopic carbon labeled alanine and isolation of the liver glycogen to determine if the increase in glycogen represents an increase in the number of carbon atoms from the alanine.

Summary. 1. The rates of absorption of D(—), L(+), and DL-alanine from the gastrointestinal tract of white rats were found to be 85, 110, and 112 mg/100 g of body weight/hour, respectively. All 3 were approximately equal in their ability to form liver glycogen, maximum values were obtained 6 hours after administration. Cortisone administration had no effect upon the rate of alanine absorption. 2. When DL-alanine was fed to rats at the same time as 5 or 2.5 mg of cortisone was injected no increase in liver glycogen was obtained over the sum of the amounts formed when each of the substances was given separately. When DL-alanine and cortisone was administered so that the peak of glycogen formation for each compound was reached at the same time then there was a significant increase in glycogen formation over the sum of the independent actions of the 2 compounds.

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Effects of Cysteamine* and Cysteine on Cardiac Output and Oxygen Content of Venous Blood. (21075)

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Considerable attention has been recently focussed on the protection against X-rays afforded in animals by both cysteine and cysteamine (= β-mercaptoethylamine = Becaptan Labaz). The radioprotective action of cysteamine has been demonstrated by Bacq and his associates(1,2) to be about 8 times as potent as that of cysteine† in C57 black mice.

Recently, Salerno and Friedell[(3,4); and discussed by Alexander Hollaender at Aarhus Conference, 1953] reported that cysteine causes tissular anoxia in dogs when given in the large amounts necessary to obtain a marked radioprotection and, in their opinion, the radioprotective effect of cysteine would be due at least partially to the induced anoxia. Thus it seemed pertinent to determine if cysteamine also causes tissular anoxia and to determine experimentally if large amounts of cysteine reduced the oxygen content of the *mixed* venous blood, as they do for the *peripheral* blood, according to Salerno and Friedell. Since the experimental procedures used for this research involve the collection of arterial and *mixed* venous blood samples, there was opportunity to study the changes in cardiac and systolic outputs and the respiratory exchanges which follow administration of cysteamine and cysteine.

Methods. Six dogs were anesthetized with chloralose. The blood pressure in the femoral artery was recorded; the heart rate could be obtained accurately at any moment. Calculations of the cardiac output were based on the well-known Fick's principle. Blood samples were collected with the usual care to avoid air contamination. Mixed venous blood was obtained directly from the right ventricle by right heart catheterization according to Cournand's technic(5), the catheter being passed along the right jugular vein. Arterial blood was taken from the femoral artery. Oxygen consumption was determined by analysis of expired air collected through a tracheal cannula in a Douglas bag; ventilation was measured in a spirometer and the gas analyses (oxygen and carbon dioxide) were carried out in Haldane's classical apparatus. Measurements were made before and after administration of the drugs, usually 5, 15, 30, and 45 minutes following injection. For each determination of the cardiac output, mixed venous blood and expired air were collected simultaneously. Since the heart rate is known at any moment, the systolic output can also be determined. Both cysteamine and cysteine were injected very slowly (in about 5 minutes) by venous route, cysteine hydrochloride having been neutralized just before injection with a normal solution of sodium hydroxide. All injections were made at one-hour intervals; cysteamine (10, 20, and 40 mg/kg) was injected first in dogs 8, 9, 10, and 11, and cysteine (20, 80, 160, and 320 mg/kg) was given first in dogs 12 and 13. Results obtained for cardiac output and systolic output are reported in Table I in which figures are calculated in percentage of the control values.

Results. 1. *Cysteamine.* Cysteamine did not significantly reduce the cardiac output. In dogs 8 and 9 there was a very slight fall of cardiac output after 45 minutes; in others, the cardiac output did not change at all (dog 10 after 5 minutes and dog 13 after 5 and 15 minutes). In 4 dogs, cysteamine produced an increase in cardiac output, ranging from 10 to 75% (dog 10 after 15 minutes). Heart rate was not affected. The changes in systolic output were similar in direction and degree to those of cardiac output. Variations in pul-

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* Bacq, Baddiley, Lipmann, Lynen, and Eldjarn have agreed to use in biological discussions the term *cysteamine* for β-mercaptoethylamine, the amine obtained by decarboxylation of cysteine (*Science*, 1954, v119, 163).

† In weight, cysteamine base (M.W.77), against cysteine HCl (M.W.158).

TABLE I. Variations in Cardiac Output and Systolic Output following Intravenous Injection of Cysteamine and Cysteine in Dogs.

Dog		Dose (mg/kg)		Control values of output (ml)	
No.	Wt (kg)	Cysteamine	Cysteine	Cardiac	Systolic
8	25	10	20	3475	24
9	20	10	80	1188	6.3
10	31	20	160	2592	16.6
11	28	20	320	1602	8.6
12	23	40	320	1872	12.3
13	24	40	320	1575	9.7

Cysteamine

Dog No.	Cardiac output (%) in indicated time* Min. 0	5	15	45	Systolic output (%) in indicated time* Min. 0	5	15	45
8	100	—	82.5	87.4	100	—	78	67.5
9	100	—	143.5	85.5	100	—	132	90.5
10	100	96	174	—	100	114	190	—
11	100	165	142	—	100	181	148	—
12	118	129	134	—	118	128	138	—
13	116	122	97	—	95	113	102	—

Cysteine

Dog No.	Cardiac output (%) in indicated time* Min. 0	5	15	30	45	Systolic output (%) in indicated time* Min. 0	5	15	30	45
8	87.4	—	84.3	77	80	67.5	—	75	57.5	62
9	85.5	—	87.8	94.9	84.3	90.5	—	93.6	100	88.8
10	174	121	126	100	—	190	112	117	100	—
11	142	56	105	165	—	148	56	105	153	—
12	100	79.9	118	—	—	100	84.6	118	—	—
13	100	94.6	116	—	—	100	95	95	—	—

* Time 0 is time of injection.

All figures are in percentage of control values (100%).

monary ventilation were as follows: an increase averaging 10-15% but never exceeding 30% occurred in all cases but one (dog 9). Oxygen consumption decreased slightly (10%) in dogs 10 and 13 and showed an average increase of 15% in the other 4 dogs. No appreciable change was observed in the oxygen content of mixed venous blood except in dog 10, where an increase of 16% was noted. In no case was a significant decrease noted; the observed slight variations of the oxygen content in the mixed venous blood are within physiological variations and accuracy of the method. No change in blood pressure was seen after cysteamine.

2. *Cysteine.* For small doses (20-80 mg/kg, dogs 8 and 9), there was little change in cardiac output: dog 8 exhibited no variation after 15 minutes and a reduction of 12% after 30 and 45 minutes while an increase of only 12% was noted in dog 9 after 30 minutes; the figures obtained in the same dog after 15 and 45 minutes were normal. The heart rate was unchanged; variations in stroke volume were parallel with those of cardiac output. Pulmonary ventilation was unaffected in dog 8, except after 15 minutes, and moderately increased in dog 9. Small elevation of the oxygen consumption was seen in dog 9, a larger one for dog 8. The figures for the oxygen content of the mixed venous blood (Table II) show that there were only physiological variations (mean value of 4.6%).

Significant decrease in cardiac output was observed when cysteine injection reached 160 mg/kg (dog 10). The fall of cardiac output was about 30% after 5 and 15 minutes following the injection of the drug and more than 40% after 30 minutes. Since there was a concomitant slight tachycardia, the systolic output dropped more than the cardiac output. Pulmonary ventilation increased moderately

TABLE II. Effects of Cysteine on Oxygen Content of the Mixed Venous Blood in Dogs (ml/Liter).

Dog No.	Dose (mg/kg)	Before cysteine	Time after cysteine (min.) 5	15	30	45
8	20	215	—	216	206	206
9	80	198	—	196	196	195
10	160	169	157	160	152	—
11	320	202	150	178	195	—
12	320	139	122	143	—	—
13	320	121	104	114	—	—

but oxygen consumption did not change. The oxygen content of the mixed venous blood decreased by 10% after 30 minutes (Table II).

Doses of 320 mg/kg of cysteine induced large decrease in the cardiac output in 2 dogs: in dog 11, the cardiac output was only 40 and 70% of normal, respectively, 5 and 15 minutes after the injection; dog 12 exhibited a smaller decrease (20% after 5 minutes) which is nevertheless significant. There was no variation in dog 13.

It is worth noting that, when it falls, the cardiac output does not remain long at low level but after some time, returns to 16-17% above the control values (30 minutes in dog 11 and 15 minutes in dogs 12 and 13). The heart rate remained unaffected and the changes in systolic output were identical with those of cardiac output. Both pulmonary ventilation and oxygen consumption increased in the 3 dogs. The blood pressure showed negligible variations except in dog 11 which, 15 minutes after injection of 320 mg/kg of cysteine, had some hypotension. The important point was that after injection of large doses of cysteine there was a significant fall in oxygen content of the mixed venous blood (Table II). This fall exceeded 13% and even reached 25% in dog 11. In all dogs, the fall in oxygen content of the blood was maximum 5 minutes after the injection of cysteine: this fall is transient since the figure for oxygen content in the blood was already rising 15 minutes after injection.

Discussion. Cysteine hydrochloride in large doses regularly induces a significant decrease in the oxygen content of the *mixed* venous blood, as already reported by Salerno and Friedell(3) for *peripheral* venous blood. It is possible that the radioprotective effects of the drug are related to this action since the maximum of desaturation in oxygen occurs just after the injection, when the radioprotective activity of the drug is also maximal. But this tissular anoxia alone cannot completely explain the protection against X-rays by cysteine because, 30 minutes after the injection, the oxygen desaturation of the mixed venous blood has returned to normal, when the radioprotection is still present, although diminished.

Cysteamine does not cause any fall of the oxygen content of the venous blood taken in the right heart; thus its radioprotective effect cannot be due to anoxia. Bacq and Alexander(6) are of the opinion that cysteamine competes with radiosensitive molecules for the oxidizing free radicals (HO_2 mainly) resulting from the ionization of water and protects a factor (or several factors) important for tissue regeneration.

Summary and conclusions. Data are submitted concerning the changes in cardiac output, systolic output, pulmonary ventilation, oxygen consumption, and oxygen content of the mixed venous blood of the right heart following intravenous administration in anesthetized dogs, of 2 radioprotectors, cysteine and cysteamine. 1. Cysteamine ($= \beta$-mercaptoethylamine $=$ Becaptan Labaz), for doses of 10-40 mg/kg, generally increases the cardiac output and the systolic output from 10 to 75% of control values. Heart rate and femoral blood pressure are unaffected. No significant change is noted in the oxygen content of the mixed venous blood. 2. The effects of cysteine are as follows: a) For doses of 20-80 mg/kg, the cardiac and systolic outputs do not change significantly; blood pressure, heart rate, and oxygen blood content remain at control values. b) Doses of 320 mg/kg in 2 of 3 dogs induce a large decrease in both cardiac output and stroke volume. In all cases, the oxygen content of the mixed venous blood is reduced, most conspicuously 5 minutes after the injection. 3. Equiprotective doses of cysteamine do not decrease the oxygen content of the mixed venous blood. 4. It is concluded: a) that the remarkable protection afforded against X-rays by β-mercaptoethylamine is not due to tissular anoxia, b) that the temporary anoxia produced by large doses of cysteine can explain only partially the radioprotective action of the substance.

I should like to express my thanks to Prof. Z. M. Bacq for suggestions, guidance, and criticism; to Prof. M. J. Dallemagne for continued interest in this work; and to E. L. Dumoulin for technical assistance.

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Effect of Chlorpromazine on Emesis after Radiation. (21076)

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Radiation sickness is an ever present danger when ionizing radiations are employed for diagnostic, therapeutic, or experimental purposes. The importance of this syndrome clinically is abundantly evident from the impressive number of references in the radiological literature. Nausea and vomiting are among the most characteristic symptoms of this disorder and numerous medications have been employed for their relief with equivocal results. Various antihistamines(1,2), vitamin (3), and hormonal(4,5) preparations, as well as other miscellaneous compounds(6,7,9) have been advocated. Recently, (dimethylamino-1-n propyl-3) - N - (2-chloro) - phenothiazine HCl (chlorpromazine) has been reported to give striking relief of nausea and vomiting in a large series of clinical patients(9). To our knowledge, no preparation has been shown to protect animals from vomiting after irradiation. In fact, such protection has only been reported(10) after destruction of the chemoceptive trigger zone described by Borison and Wang(11). Since chlorpromazine protects dogs against other emetic stimuli acting upon this area (apomorphine(12), hydergine(13), motion(14)), it was of obvious interest to test its effectiveness against radiation emesis as well.

Methods. As controls, 12 normal mongrel dogs, weighing from 7 to 13 kg, were fed commercial dog food and 30 minutes later irradiated with 800 r. A Picker deep therapy x-ray unit was employed with 260 KVP and 18 MA. The external filtration was 1 mm Al and 0.50 mm cu plus 0.25 mm cu inherent filtration. The target-to-object distance was 118 cm and the dose rate 13 r/minute in air. The dog received half the radiation dose on one side of the body, at which time he was turned and given the remainder of the dose. After irradiation, the dogs were observed from 4 to 6 hours, unless vomiting occurred earlier. Another group of normal dogs within the same weight group received subcutaneously 5 or 10 mg of chlorpromazine† /kg body weight freshly prepared in 0.9% saline. They were immediately fed and 30 minutes later irradiated with 800 r as described above. On one occasion, a solution of chlorpromazine was not used for 2 hours, during which time it assumed a slightly brown color. To test the effect of such standing, 5 dogs received 10 mg/kg of a chlorpromazine solution allowed to "age"

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† Generously supplied by Smith, Kline and French Laboratories, Philadelphia, Pa.

TABLE I. Radiation Emesis in Dogs after Chlorpromazine or Cysteinamine.

Medication	Dose, mg/kg	Dogs vomiting/dogs tested	Latent vomiting period after radiation (min.)	Survival (days)
0	—	12/12	32.4	3.8
Chl.*	10	2/10	72.5	5.7
" ("aged")	10	4/ 5	66.2	5.8
"	5	4/ 5	69.8	4.3
Cys.* (before rad.)	200†	5/ 5	25.0	5.0
" (after ")	"	4/ 5	22.3	5.2
" (during ")	"	8/11	55.1	6.2

* Chl. = Chlorpromazine; Cys. = Cysteinamine.
† Total dose given to all dogs regardless of weight.

for 2 hours at room temperature in an open flask. Since cysteinamine has been reported to control vomiting clinically after x-ray therapy(8), it was included in the present investigation for comparative purposes. Its ability to prevent vomiting in animals has not been previously investigated. At first 5 dogs received 200 mg of cysteinamine‡ intravenously immediately prior to the start of irradiation. Injection at this time has been shown(15) to allow greatest survival in smaller animals. The duration of action of cysteinamine is short and injections *after* irradiation have failed to increase or prolong animal survival. Nevertheless, clinically, intravenous injections have been reported to relieve or eliminate the symptoms (vomiting, nausea, diarrhea, etc.) resulting from prior irradiation. For this reason, a second series of dogs(5) were given 200 mg of cysteinamine intravenously immediately *after* irradiation. Finally, a third group(10) received the same dose intravenously *during* irradiation. The dogs received 400 r whole body radiation, were injected with cysteinamine and then immediately received the remaining 400 r. It was hoped by these variations in timing that optimal conditions would be realized in one group.

Results. The relative effectiveness of the various preparations is compared in Table I. It can be seen that freshly prepared chlorpromazine (10 mg/kg of body weight) affords good protection against vomiting after irradiation. All controls vomited within 2 hours after radiation was completed and 9 of the 12 within 30 minutes. With chlorpromazine, only 2 out of 10 vomited, one after 56 and the other after 89 minutes. However, when the solution of chlorpromazine in saline was allowed to stand for 2 hours before injection, all but one dog vomited after radiation although the latent vomiting period was increased. The lowered dose of chlorpromazine (5 mg/kg) gave almost identical results, with little, if any, protection against vomiting but an apparent increase in the latent period. Cysteinamine given before or after 800 r x-radiation neither prevented nor delayed the vomiting beyond that of the controls. However, of 11 dogs receiving cysteinamine immediately interposed between 2-400 r exposures, 3 failed to vomit and the average latent vomiting time of the remaining animals was increased.

The apparent increased survival times with both chlorpromazine and cysteinamine is suggestive, but the series are too small to permit conviction on this point.

Discussion. The effective dosage of chlorpromazine used here was larger than that necessary to prevent vomiting in dogs after the injection of apomorphine(16,17), ergot(16), or after swinging(17). As has been pointed out previously(9,16,17), subcutaneous injections of 10 mg/kg produces some degree of depression. This raises the question whether the protection noted was due to a specific effect on the chemoceptive emetic trigger zone or a non-specific effect due to depression of the vomiting center. Glaviano and Wang(13) have pointed out that 2.0 mg/kg or more of chlorpromazine significantly increases the threshold to oral $CuSO_4$. This indicates a depression of the reticular vomiting mechanism. It is probable, therefore, that the doses employed in the present study produced some central depression. Whether this depression was adequate to account for the decreased sensitivity to radiation cannot be stated. However, preliminary results utilizing other depressants have failed to demonstrate a comparable protection against radiation vomiting. The lowered effectiveness of solutions allowed to stand for 2 hours is not in accord with reports on its stability for 24 hours(9). No explanation is available for this discrepancy.

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‡ Generously supplied by E. R. Squibb & Sons, New Brunswick, N. J.

The apparent increase in survival time after chlorpromazine requires confirmation with larger series of animals. Studies with mice and guinea pigs have failed to confirm this prolonged survival following x-radiation(18). Additional dogs have not yet been studied.

Summary. Chlorpromazine-(dimethylamino-1-n propyl-3) - N - (2-chloro) - phenothiazine HCl in doses of 10 mg/kg injected subcutaneously protected dogs against vomiting after 800 r x-radiation. Injections of 5 mg/kg were ineffective as were 10 mg/kg prepared from solutions allowed to stand for 2 hours at room temperature before injection. Cysteinamine (200 mg intravenously) had no or questionable protection whether given before, during, or after radiation. Increased survival time was noted with both chlorpromazine and cysteinamine. The mechanism of protection is discussed.

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Esterification of Soybean Sterols *in vitro* and their Influence on Blood Cholesterol Level. (21077)

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It has been reported recently(1,2) that the feeding of soybean sterols to rats resulted in a lowering of the blood and lymph cholesterol. The decrease was mainly in the ester fraction. An explanation offered(2) for these effects is that the plant sterols interfere with the esterification of cholesterol in its passage from the lumen of the intestine to the lymph. In connection with studies being carried out in these laboratories it has been demonstrated that the enzyme cholesterol esterase and bile salts play an important role in the esterification of cholesterol and in cholesterol absorption(3,4). It became of interest to determine

TABLE I. Blood Cholesterol Levels of Rats Fed Soybean Sterols in a Basal Diet Containing Cholesterol, Bile Salts and Fat.

Group	No. rats	Days on diet	Soybean sterol content, %	Whole blood cholesterol,* mg % Free	Ester	Total
	48	Pre-diet		75.9	34.0	109.9
AS	8	7	.0	108.9	85.4	194.3
	8	14	.0	114.0	144.0	258.1
	8	21	.0	116.2	236.8	353.0
AT	8	7	.5	108.8	84.2	193.0
	8	14	.5	99.9	133.4	233.3
	8	21	.5	93.5	186.4	279.9
AU	8	7	1	98.2	60.2	158.4
	8	14	1	96.2	125.7	221.9
	8	21	1	80.2	124.5	205.1
AV	8	7	2	78.2	73.7	151.9
	8	14	2	86.2	92.7	178.9
	8	21	2	73.0	135.8	208.8
AW	8	7	3	84.0	56.4	140.4
	8	14	3	80.1	85.4	165.5
	8	21	3	77.6	97.6	175.2
AX	8	7	4	89.8	45.7	135.5
	8	14	4	81.3	75.8	157.1
	8	21	4	77.8	64.1	141.9

* Avg stand. dev. of total blood cholesterol was ± 4 mg % in pre-diet period; during experimental period, ± 36 mg %.

if soybean sterols inhibit the marked increase in blood cholesterol of rats which results from a diet containing cholesterol, fat, and bile salts(4) and also if soybean sterols are esterified by pancreatic cholesterol esterase. Such findings might supply additional support for the suggested mechanism of action of dietary soybean sterols on the blood cholesterol.

Methods and materials. In vivo experiments. Forty-eight rats (male and female) from our stock colony, weighing 175-200 g and raised on Purina pellet chow were used. The animals were housed in separate cages and given water *ad libitum.* Following a control period on pellet chow during which the blood cholesterol fractions were determined, the rats were divided into 6 equal groups. The animals were pair-fed according to the following technique: after the first day all rats in all groups received the amount of food ingested the previous day by the rat having the lowest food intake. Each group received the basal diet to which was added either 0, (control group) .5, 1, 2, 3, or 4% soybean sterols. The basal diet had the following composition: 20% casein, 22% starch, 23% glucose, 25% olive oil, 5% Hubbel-Mendel-Wakeman salt mixture, 2% ruffex, 2% cholesterol, 1% sodium taurocholate, and adequate amounts of the crystalline vitamins. The soybean sterols were added at the expense of the starch and glucose. Blood samples were collected directly from the tail vein in .2 ml pipettes and placed in 1:1 acetone alcohol mixture for cholesterol analysis(5). *In vitro experiments.* Substrate mixtures were prepared as described earlier(6) with the exception that 250 mg blood albumin was used in lieu of egg albumin. Cholesterol-oleic acid and soybean sterol-oleic acid were used as the substrates for esterification. One of the important aspects of studies on cholesterol esterase is the uniformity and stability of the emulsification of substrates. Our criterion(6) for uniformity and stability of emulsification has been that the total cholesterol content of duplicate samples removed from the enzyme digests at different times should agree within the limits of error of the analytical method for determining cholesterol. A homogenate of hog pancreas(7) was used as the enzyme source. The reaction time was 1 hour. All calculations were based on the amount of free cholesterol and soybean sterol present at zero time and

TABLE II. Esterification of Soybean Sterols and Cholesterol with Oleic Acid by Pancreatic Cholesterol Esterase. Bile salt, sodium taurocholate at the optimum for each substrate concentration(3). pH of digests, 6.1. Oleic acid conc., 3 equivalents in terms of cholesterol. Enzyme, 1 ml 10% hog pancreas homogenate.

Soybean sterol, mg/digest	Esterification, mg/esterified/hr	Cholesterol, mg/digest	Esterification, mg/esterified/hr
14	7.0	14	7.8
29	15.2	29	17.2
43	19.7	39	22.0
55	21.8	55	31.5
86	26.8	82	34.0
86	.0*		
86	.0†		

* No bile salts present in enzyme digest.
† No fatty acid " " " " .

at the end of the incubation period. Soybean sterols were determined in the same manner as cholesterol, *i.e.* digitonin precipitation followed by the Lieberman-Burchard color reaction(5) and comparison with standard solutions of soybean sterols.

Results. Table I, Group AS, shows the effect of the basal diet containing 2% cholesterol plus 1% sodium taurocholate on the free, total and ester cholesterol of blood. Both total and ester fractions increased markedly and after 21 days on the basal diet; the total and ester fractions were 353 and 237 mg % respectively. The addition of soybean sterols to the diet (Groups AT-AX) resulted in a lesser rise of the blood cholesterol, especially in the ester fraction, than occurred in the group not receiving soybean sterols. The higher the concentration of soybean sterols in the diet, the greater was the difference. At a concentration of soybean sterols of 4%, (Group AX), the total blood cholesterol level after 3 weeks was 142 mg % which is 148% lower than the group not receiving soybean sterols and only 30% above the control level when the animals were on pellet chow. Thus the addition of 4% soybean sterols practically completely inhibited the increase in the blood cholesterol, which occurred in the animals on the basal diet.

Since esterification(9) is presumed to play an important role in the absorption of cholesterol, it was desirable to ascertain if soybean sterols were esterified *in vitro* by pancreatic cholesterol esterase and, if so, under what conditions. Table II shows the enzymatic activity obtained with soybean sterols and cholesterol as substrates. Both sterols are esterified with oleic acid in the presence of the enzyme. However, cholesterol is esterified somewhat more rapidly. It was also observed that bile salts and fatty acid are required for the esterification of soybean sterols. Similar findings have been reported with respect to cholesterol(6).

Discussion. Plant sterols have been used to lower the blood cholesterol of animals and humans(1,10), but their practicability seems questionable because of the necessity of feeding relatively large amounts. The results of the feeding experiments described above give support to the suggestion(2) that soybean sterols interfere with the absorption of cholesterol. It is also shown in this study that plant sterols and cholesterol are esterified under the same conditions by pancreatic cholesterol esterase *in vitro*. Hence, since the enzyme does not show any specificity towards these substrates, it may be presumed that it acts similarly in the intestine. It is probable that the soybean sterols compete with the cholesterol for the enzyme, bile salts, and fatty acids. That would impair the absorption of cholesterol by inhibiting its esterification. It is interesting to note that even though plant sterols are esterified, they are not absorbed(8).

Summary. Soybean sterols inhibit the increase in blood cholesterol of rats receiving a diet containing 2% cholesterol and 1% sodium taurocholate. The degree of inhibition increased as the soybean sterol concentration of the diet was increased. It was also observed that pancreatic cholesterol esterase catalyzes the esterification of soybean sterols albeit at a slower rate than with cholesterol. Bile salts and a fatty acid source are required for their esterification. The mechanism of action of soybean sterols in inhibiting cholesterol absorption is discussed.

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Serum Lipoproteins in Experimental Diabetes. II. Action of Heparin *in vivo* on Lipoproteins of Depancreatized Dogs.* (21078)

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Clearing of lipemic serum by heparin was first observed by Hahn(1) and by Weld(2). Further study of the phenomenon demonstrated that heparin combines with a "co-protein" supplied by normal plasma to form a "clearing factor"(3-5), capable of causing profound changes in the lipid and lipoprotein composition of normal human and animal serum and of the serum of patients with atherosclerosis or hypercholesterolemia(6-10).

The object of this investigation was to compare the changes caused by heparin in the serum lipoproteins of normal lipemic dogs with those caused in the serum lipoproteins of diabetic lipemic dogs.

Materials and methods. Two normal dogs, kept on a commercial dog food diet, were fasted for about 20 hours and, after taking a control sample of venous blood, fed a meal consisting of 25 g of lard, 2 egg yolks (dog W), and a small amount of commercial dog food. Serum samples were obtained 3 hours after the meal, at which time heparin sodium (2 mg/kg) was injected intravenously;‡ other blood samples were obtained 10 minutes (dog W) and 30 minutes after the injection. Five depancreatized dogs were allowed to develop mild to severe ketosis and lipemia by the withdrawal of insulin. After securing a fasting blood sample, heparin was injected intravenously and another blood sample obtained 30 minutes after the injection. Serum was separated by centrifugation and analyzed for total cholesterol and lipid phosphorus according to the methods of Bloor, Pelkan and Allen (11) and of Horwitt(12), respectively. Serum aliquots were subjected to electrophoresis on filter-paper, following a previously described technic(13,14) and the cholesterol and lipid phosphorus content of the resulting fractions was determined. A semi-quantitative pattern of the total lipid distribution along the strip of filter-paper was obtained by staining the strip with Sudan III(13) and measuring the density of the stain with a spectrophotometer at a wave length of 520 mμ. A curve was obtained by plotting color density against the distance along the strip.

Results. Normal dogs. The ingestion of a fatty meal caused a small rise in the total cholesterol and lipid phosphorus content of serum. This was accompanied by a rise in the slow-migrating lipoprotein fractions (β_1, β_2), while the cholesterol and lipid P content of the fast-moving components decreased slightly. These changes were not statistically significant. On the other hand, the total lipid content of the non-moving band ($\beta_{2\gamma}$) increased sharply, probably as a result of neutral fat load. This is indicated also by the intensity of the color in the strips stained with Sudan III (Fig. 1). The administration of heparin

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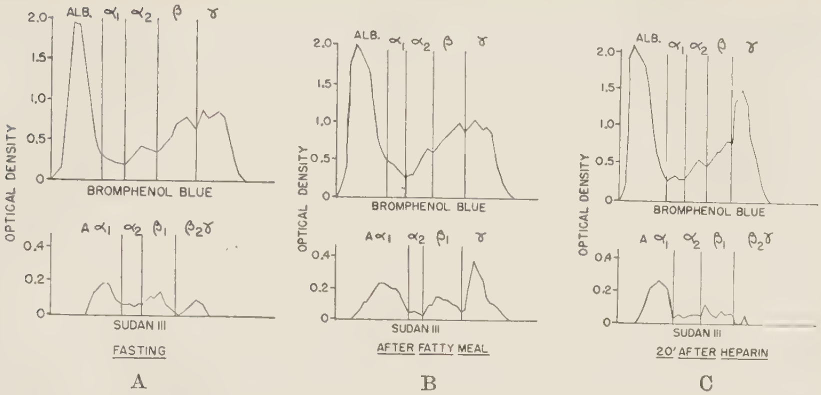

FIG. 1. Paper electrophoresis pattern of proteins (upper curve) and lipids (lower curve) of normal dog serum. Galvanometer readings. Instrument set at zero with unstained portion of strip. A: fasting; B: after fatty meal; C: after heparin.

caused a complete clearing of the lipemia accompanied by a reversal of the changes described above in a matter of minutes. The Sudan III peak in the $\beta_2\gamma$ zone disappeared (Fig. 1); the cholesterol and lipid phosphorus content in the whole serum and in the slow-moving fractions decreased slightly while the lipid content of the albumin-α_1 fraction increased slightly. The lipid content of the α_2-fraction increased somewhat immediately after the injection of heparin in dog W, but a few minutes later, returned to a level lower than normal. In order to detect possible lipoprotein components migrating faster than albumin, the zone of the strip ahead of the latter was also analyzed; while in one case (dog Y), no lipids were detected in this zone, in the other case (dog W), there appeared small amounts of lipids soon after the injection of heparin.

Diabetic dogs. The following changes were observed in dogs with diabetic lipemia following the injection of heparin (Table I and Fig. 2): 1) varying degrees of clearing of the

TABLE I. Effect of Fatty Meal and of Heparin on Cholesterol and Lipid Phosphorus Fractions in Normal and Diabetic Dogs.

			Cholesterol fractions, mg %						Lipid P fractions, mg %					
	Dog	Time	Total	F	α_1	α_2	β_1	β_2	Total	F	α_1	α_2	β_1	β_2
Normals	Y	Fasting	230	—	185	24	11.5	9.5	11.4	—	9.5	.9	.75	.25
		3 hr after M*	234	—	180	20	17	17	11.5	—	9.3	1.1	.6	.5
		20′ after H*	198	—	177	6	11	4	11.6	—	10.4	.7	.4	.1
	W	Fasting	201	1	157	11	15.5	16.5	9.9	—	8.2	.5	.6	.6
		3 hr after M	202	2	137	15	21	27	10.6	.1	8.0	1.0	.8	.7
		9′ after H	192	10	140	18	10	14	10	.7	7.1	1.2	.5	.4
		20′ after H	185	5.5	148	2.5	12	17	10	.3	8.3	.5	.55	.35
Diabetics	P	Before H	538	—	390	92	32	24	11.3	—	7.7	1.9	1.1	.6
		30′ after H	492	—	406	66	11	9	10.4	—	8.2	1.5	.65	.05
	R	*Idem*	692	20	380	96	126	70	12.6	.2	6.5	1.9	3.0	1.0
			648	243	165	78	82	80	13.4	6.15	3.3	1.2	1.5	1.25
	K_1	”	418	40	264	70	27	17	12.2	1.6	7.6	1.9	.8	.3
			420	169	142	54	34	21	12.1	5.2	4.3	1.2	1.2	.2
	Z	”	319	9	247	18	26	20	7.9	.2	5.5	.4	1.2	.6
			320	21	241	13	21	24	7.3	.4	5.3	.35	.9	.35
	K_2	”	354	115	90	78	40	31						
			344	163	62	33	37	49						
	U	”	444	91	281	29	25	18						
			428	250	103	29	21	25						

* M = Fatty meal. H = Heparin I.V.

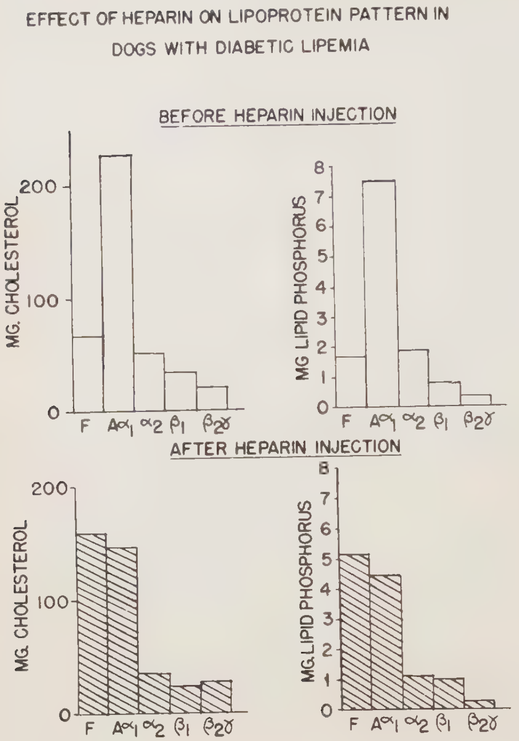

FIG. 2. Distribution of cholesterol and lipid P in main lipoprotein fractions of dogs with diabetic lipemia.

serum which, in the case of dog P, appeared to be nearly complete; 2) a moderate decrease in total serum cholesterol and lipid phosphorus; 3) a slight change in total serum lipids; 4) a marked change in the lipid distribution characterized by a sharp increase in the fraction moving faster than albumin. Small amounts of this fraction (which, for the sake of brevity, will be called fraction "F") are sometimes present in the serum of diabetic dogs, however, after the injection of heparin, it may account for as much as 40% of the total serum cholesterol and lipid phosphorus (Table I). Fraction F can be seen also in the free-electrophoresis pattern of the serum of one dog studied by this method (Fig. 3);§ in this case (Dog U), fraction F represented 11% of the total serum protein area. The cholesterol/lipid P ratio of fraction F closely resembles that of the albumin-α_1 fraction. Though a complete analysis of its composition was not made, the high lipid content of fraction F is noteworthy; 5) the albumin-α_1 fraction decreased in amounts which appear to be equivalent to the rise in fraction F; and 6) the slow-moving $\beta_2\gamma$ fraction changed very little or not at all, most often increasing slightly rather than decreasing.

Discussion. The mechanism of action of the clearing factor produced after injection of heparin is not fully understood. It is now generally agreed that the phenomenon of clearing is accompanied by a conversion of lipoproteins of low density, low electrophoretic mobility and large molecular size into smaller, faster-migrating ones(9,15): The results obtained in normal dogs with alimentary lipemia which are very similar to those obtained in man(10,15,16), are consistent with this conversion theory. Still matter for speculation is how this conversion is brought about (17-19). It is possible that the highly mobile fraction F may play a role in the clearing phenomenon, perhaps as an albumin-like co-factor(5). This is suggested by: 1) the sharp increase in fraction F after heparin;

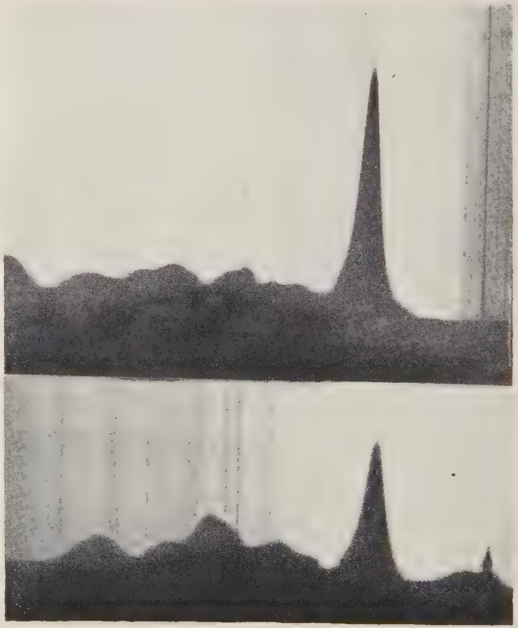

FIG. 3. Free electrophoresis pattern (ascending limb) of normal dog serum before (A) and after (B) heparin injection. Note fraction F at the right of the albumin peak. Left to right. Veronal buffer pH 8.6. Ionic strength 0.1.

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§ Free electrophoretic determinations were performed through the courtesy of Doctor Steelman of Armour Laboratories.

2) its high lipid content; and 3) its high mobility indicating small molecular size with a net increase in electrical charge and, most likely, increased solubility. The appearance of an extra-boundary fraction comparable to our fraction F was observed recently by Lever and co-workers(20) studying human sera by means of free electrophoresis. The authors suggest that the new fraction might be the results of a combination of heparin with a lipoprotein, or of the splitting of large lipoprotein molecules caused by the lipolytic action of the clearing factor. The presence of fraction F in diabetic dogs untreated with heparin and its appearance in normal dogs after a fatty meal as well as after heparin suggests the alternate possibility that, at least in the dog, fraction F might be a normal serum component, and that the differences between normal and diabetic animals in their response to heparin might be a quantitative rather than a qualitative one. Fraction F appears to be related to fraction α_1, as indicated by the reciprocal relationship of their lipid content. It is possible that the partial clearing observed in the serum of diabetic lipemic dogs after heparin may be due, at least in part, to an increase in this lipid carrier of relatively high solubility. The residual turbidity is probably due to fraction $\beta_2\gamma$ which, unlike in the normal animal, does not appear to be affected by heparin, suggesting a derangement of the clearing phenomenon in most diabetic decompensated dogs.

Summary. Heparin causes a clearing of the lipemic serum of normal and, less, of depancreatized dogs. In normal animals with alimentary hyperlipemia, this clearing is associated with a decrease in the lipid content of the slow-moving lipoprotein fractions, while in the diabetic lipemic dogs, the outstanding change is the appearance of, or sharp increase in, the amount of a fraction "F" migrating faster than albumin and containing large quantities of lipids. The possible significance of these results is discussed.

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A Rapid Method for Isolation of Mixed Conjugated Bile Acids From Bile.* (21079)

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In the course of studies on the comparative biochemistry of cholesterol metabolism, it became necessary to isolate conjugated bile acids from the bile of several species of animals. Published procedures are based on the use of either single-stage extraction or countercurrent distribution(1). The former method does not readily yield a pure product, while the latter requires elaborate equipment. In this communication, a simple adsorption technic for the rapid isolation of the compounds is presented.

Method. The sample of bile is mixed with 20 volumes of absolute ethanol, and the precipitated salts and proteins are removed by filtration. The filtrate and washings are evaporated to dryness *in vacuo,* with temperature kept below 40°. The brown, gummy residue is taken up in a small volume of methanol, the solution filtered if necessary and applied to an alumina column. (The amount of alumina should be at least 60 times that of the residue.) The column is washed with methanol, and the methanol is collected in fractions. The bile pigments are completely retained on the top of the column, while bile acids, neutral steroids, and fats wash through rapidly. Occasionally the retention of bile pigments is not complete, and when this occurs, larger quantities of alumina in a wide column of high flow rate should be used. The first fraction collected often has a yellow tinge which, however, does not interfere with the subsequent steps. Each fraction is evaporated to a small volume, and excess anhydrous ether is added. The conjugated bile acids precipitate out as a white, flocculent powder, while the neutral steroids, fats, phospholipids, and unconjugated bile acids (if present) remain in solution. (Failure to employ anhydrous ether may result in a gummy, hygroscopic precipitate of bile acids.) Elution of the column with methanol is discontinued when fractions no longer yield precipitates upon the addition of ether. The conjugated bile acids are then filtered off, washed with ether, and dried. It is desirable to reprecipitate the isolated bile acids once or twice from methanol-ethyl ether mixtures as described above.

TABLE I. Recovery of C^{14}-Labeled Contaminants in Conjugated Bile Acids Isolated from Rat Bile. 10-ml samples of rat bile were used. The column measured 12 mm × 50 mm. Total volume of methanol collected was 150 cc. This was evaporated to about 8 cc and then diluted to 60 cc with ethyl ether to precipitate bile acids.

C^{14}-labeled compound added*	No. of times reprecipitated	% C^{14} recovered	Yield of bile acids, mg
Cholesterol	2	.1	82
Tripalmitin	1	1.5	98
Octanoic acid	2	1.4	134
Glucose	2	.2	87
Succinic acid	2	2.9	81

* Less than one mg of the labeled compound was added.

In order to check the procedure, bile acids were isolated, as described above, from 10-ml samples of rat bile to which had been added trace amounts (less than 1 mg) of a number of C^{14}-labeled compounds which might be found in bile. The extent to which the added C^{14} was recovered in the isolated bile acids is shown in Table I. Since bile ordinarily contains very small amounts of these compounds, the contamination from them can be neglected for most purposes.

Ordinarily most bile acids in bile are conjugated, and since bile is slightly alkaline, these acids will be present as their sodium salts. If for some reason large amounts of free (unconjugated) bile acids are present, these will also be precipitated by ether as sodium salts. These free bile acids can, however, be eliminated by first acidifying the bile sample and then following the procedure as described. The subsequent addition of ethyl ether will then precipitate only the conjugated

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* Aided by a grant from the U. S. Public Health Service.

TABLE II. Yields of Conjugated Bile Acids from Various Species.

Species	Yield of bile acids per 10 ml bile, mg
Rat	80–100
Rabbit	225
Chick	240
Goose	1800*
Frog	650

* Bile partially dehydrated.

bile acids leaving the free acids in solution.

We have applied this method to the bile of several species (Table II) with uniformly good results. In all cases, the isolated bile acids were chromatographed in collidine-water-NH_3(2), and compared with chromatograms of whole bile. The isolated bile acids were always chromatographically identical with the conjugated bile acids of whole bile. It should be noted, however, that since conjugated bile acids are slightly soluble in methanol-ethyl ether mixtures, this method cannot be used for the quantitative recovery of conjugated bile acids in bile.

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Observations on Effects of X-Irradiation on *Achromobacter fischerii*.*

(21080)

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Bacterial luminescence offers a unique opportunity for studying the effect(s) of x-irradiation on the living cell. Due to the interaction of irradiation and cellular substance, an almost immediate visual indication of alteration in the biochemical processes of the cell is observed at proper dosage levels. The most recent review on the effect of x-irradiation on luminescent bacteria [Harvey(1)], has called attention to the need for more extensive investigation of this subject.

Lorenz and Henshaw(2) have studied the survival of *Achromobacter fischerii* after x-irradiation and Whipple(3) has reported some effects of x-rays on bacterial luminescence. Unfortunately, the experimental technics differ, and no correlative evaluation of the relative sensitivities of bacterial luminescence and survival can be made from this material. It has been the purpose of this study to investigate the effect of graded doses of x-irradiation on bacterial luminescence relative to survival of the microorganisms.

Materials and methods. The test organism used for this study was *Achromobacter fischerii*,‡ cultured in Farghaly's basal media (4) supplemented with 200 mg purified casamino acids per liter. The cells were grown at room temperature on a horizontal oscillating machine for a period of 18 hours and then harvested in the cold by centrifugation. The centrifuged cells were washed with a cold NaCl-PO_4 buffer(1), and resuspended in a suitable volume of buffer to give a cell concentration of approximately 5 million cells per milliliter. This cell concentration was used as the standard. Aliquots of the standard cell suspension were pipetted into small round glass cups and exposed to x-irradiation delivered by a 250 kv Westinghouse unit with a 15 ma output at a distance of 14 cm using 1 mm Al and 0.5 mm Cu filters. The dose rate was 545 r per minute as measured with a Victoreen r-meter The liquid depth of the exposed bacterial suspension was 5 mm. After irradiation, samples of both the test and control suspensions were taken to determine the

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* Supported in part by the U. S. Atomic Energy Commission.

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‡ The original culture of this organism was kindly supplied by Dr. Frank H. Johnson, Biology Department, Princeton University.

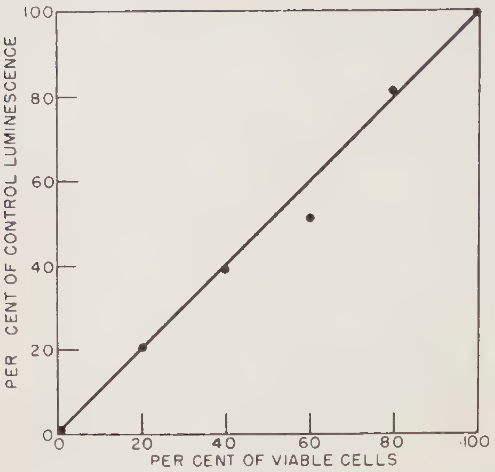

FIG. 1.

x-irradiation effects on luminescence and viability. *Luminescence* was determined on both the test and control samples after buffered 15% dextrose had been added to the samples to give a final concentration of one percent dextrose. Three aliquots of each sample were then taken for determinations. The luminescence was measured by means of a GE 931-A electron multiplier tube incorporated within a small constant temperature (26°C) apparatus which maintained a constant rate of agitation for the tubes containing the suspensions. Aeration of the suspensions was facilitated by means of a small glass bead in each tube. *Viability* was determined by taking 1 ml aliquots of both test and control cultures and diluting with buffer through a series of ten-fold steps. One-tenth ml for each dilution was placed on each of three agar plates and smeared evenly over the surface with a sterile bent glass rod. The cultures were incubated for a period of 36 hours and then counted. The survivals are listed as a log percentage of the controls. The medium for plate counts was essentially the same as described by Harvey(1) with the exception that yeast extract and $NaHCO_3$ were substituted for beef extract and $CaCO_3$ respectively, to enhance the transparency of the medium. The carbonate was added to the medium after autoclaving and just prior to pouring the plates.

Results. The effect of various percentages of dead cells in a standard bacterial suspension on the percentage luminescence of this mixed dead and live cell suspension was studied. In this manner the lethal effects of various dose levels of x-irradiation could be simulated and a quantitative relationship between luminescence and viable cell count obtained. For this purpose a suspension of formalin-heat-killed cells was used as a diluent for the standard suspension. This killed suspension first was washed 5 times with buffer and then diluted to a turbidity equal to that of the standard cell suspension. Dilutions of the viable standard cell suspension were then made with the killed cells as noted in Fig. 1. By keeping the turbidity constant, while varying the relative number of dead cells, conditions supposedly existing in x-irradiated cultures could be approximated.

To test the above results, a standard cell suspension was exposed to doses of x-irradiation ranging from 500 to 10,000 r. Aliquots of the irradiated and control suspensions were then plated out after exposure to determine the sensitivity of the organism to graded doses of x-ray. Aliquots were also taken from these samples for measurements of luminescence to find the immediate effects of the irradiation on this system. The results shown in Fig. 2 represent luminescence 2 hours after x-irradiation at which time the ratio of the test to the control had become constant in all groups. It is apparent that x-irradiation at all dose levels affected the survival of the cells, while the effect on the luminescence did not become apparent until the dose was increased to 5000 and 10000 r. A comparison of the log percentage survival and the log percentage luminescence after treatment with the higher x-ray doses suggests that a large proportion of the cells unable to multiply after x-irradiation are still capable of carrying out the necessary metabolic processes required for the production of luminescence. Using the results in Fig. 1 as a reference, the *calculated viability* was obtained, and it was noted that the luminescence persisting two hours after x-irradiation with 5000 r was equal to a survival of approximately 56% on the control suspension, while the luminescence of those exposed to 10000 r was equivalent to a 17% survival.

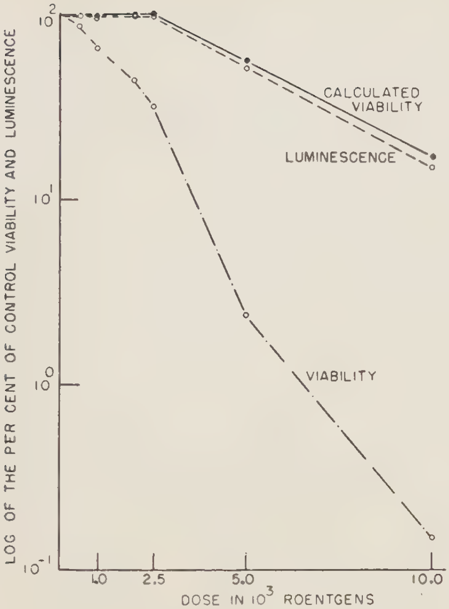

FIG. 2.

The measurements of reproductive ability of the suspensions were only 1.24 and 0.115% survivals respectively, indicating that the capacity of the cell for multiplication after exposure to x-irradiation is increasingly more sensitive to exposure than is the system for luminescence. Giese(5) has observed that although low dosages of ultra-violet light have a sterilizing effect on *A. fischerii,* such treatment does not immediately impair the respiration or luminescence. Likewise, Billen *et al.* (6) have found bacterial respiration after x-irradiation to be far greater than could be expected on the basis of viable cell count.

Inasmuch as bacterial luminescence in the intact cell is dependent on oxygen concentration(7) as well as the proper functioning of the respiratory system(1) and cell extracts require at least coenzyme I(8) and riboflavin phosphate(9), it can be assumed that a large proportion of the sterilized cells is able to carry on metabolic activities for a measurable length of time after x-irradiation.

Summary. It has been observed that the reproductive mechanism of *A. fischerii* is more sensitive to x-irradiation at all measured dosage levels than is the luminescent system of this organism. These findings indicate that cells sterilized by x-irradiation are capable of metabolic activity for a measurable length of time after exposure.

Grateful acknowledgement is made to Mr. G. Westinskow and Mr. D. Taysum for designing and assembling the electronic and mechanical equipment used in this work.

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Lyophilization of Poliomyelitis Virus. Heat Inactivation of Dry MEFl Virus.* (21081)

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Few attempts to dry the poliomyelitis viruses have been reported. Landsteiner and Levaditi(1), Römer and Joseph(2), Landsteiner, Levaditi, and Pastia(3) have indicated success in drying infective monkey cords or cord suspensions over KOH, *in vacuo,* and over P_2O_5, respectively. Each of these reports involved testing of the dried material in one monkey, and in no case were virus titrations before and after drying carried out. Epidemiological considerations based on dry virus in nature were indicated principally by Römer and Joseph(2).

In order to measure accurately some of the physical properties of these viruses, *e.g.,* infective particle size as determined by deuteron bombardment(4), it is essential that the material be dried first. This report concerns a method of drying MEFl (Type 2) and Leon (Type 3) poliomyelitis viruses *in vacuo,* as well as the heat inactivation of the former.

Materials and methods. Virus strains. The MEFl baby mouse adapted strain of Casals *et al.*(5) in its 138th passage was used throughout. A large pool of 50% baby mouse brain in water† was prepared, carefully homogenized, divided into aliquots, frozen and stored at –70°C until needed. It was diluted before drying to a 10 or 20% suspension with any of the media to be described. Spinal cords (5% suspension) from adult mice infected with the mouse adapted Leon virus(6, 7) in its 90th passage were also used.‡ *Virus titrations.* These were done in adult Swiss albino mice by the intraspinal technic of Habel and Li(8). Ten-fold virus dilutions were prepared in saline and 8 mice per dilution were inoculated with 0.02 ml each. They were observed 10 days. The PD_{50} was estimated by the method of Reed and Muench (9), and the titers given below were calculated per inoculum. *Lyophilization.* The material to be dried was shell frozen in small Pyrex ampules in 0.2 ml amounts. Evacuation was effected by a pump with the aid of a condenser immersed in a mixture of methyl cellusolve and dry ice, the entire set-up being kept at room temperature. With the small volumes used, drying was complete in 6 to 8 hours, or 1½ times the time required for all visible moisture to disappear from the outside of the tubes. Later it was found that the procedure was aided by keeping the material cold while it was drying. Thus a mixture of crushed ice and rock salt surrounded the tubes. After drying was complete, dry air was admitted and the tubes were sealed. In no case was it attempted to seal the tubes *in vacuo. Lyophilizing media.* In checking routine bacteriological media for their possible protective effect on dry poliomyelitis virus, it was found that peptone water (1% Bacto-peptone§ and 0.5% NaCl in water) and Na thioglycollate broth§ were relatively successful as compared with water or saline alone. Various concentrations in water of peptone (from 2 to 30%) at pH 7, Na thioglycollate (2.5 and 5%) at pH 8, 0.5% gelatin, 3% cysteine • HCl neutralized to pH 5 with NaOH, and beef heart infusion broth were prepared. Each of these was added to the 50% baby mouse brain in a volume that yielded a 10 or 20% brain suspension. When Leon virus was dried, 1 part of a 10% cord suspension that had been frozen and thawed twice with subsequent centrifugation was added to 1 part of 30% peptone. *Reconstitution of the dry virus.* The dry material was reconstituted either in water or 30% peptone

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* Supported by the Office of Naval Research.

† Demineralized water was used throughout.

‡ We are grateful to Dr. J. Casals for providing the MEFl virus and to Drs. C. P. Li and M. Schaeffer for the Leon virus.

§ Difco Laboratories, Detroit, Mich.

TABLE I. Results of Drying MEF1 Poliomyelitis Virus in Various Media.

% baby mouse brain	Drying medium*	Dried in cold‡	Reconstituting medium	Virus titer†		% virus survival
				Before drying	After drying	
10	10% peptone	Yes	Water	5.9	5.4	32
20	30% ”	”	”	6.0	5.5	32
10	5% Na thioglycollate	”	”	5.0	4.5	32
10	5% peptone	”	”	5.9	5.0	13
10	10% ”	No	”	5.7	3.5	.6
20	10% ”	”	”	5.9	4.4	3
10	Peptone water	Yes	”	5.7	4.6	5
10	*Idem*	No	”	6.0	4.3	2
10	Thioglycollate broth	”	”	6.0	4.3	2
10	0.5% gelatine	Yes	”	5.7	3.5	.6
10	Cysteine	No	”	5.3	3.4	1.3
20	”	”	”	5.3	4.0	5
10	Water	”	”	6.0	<3.0	<.1
20	”	”	”	5.9	<3.0	<.1
20	”	Yes	”	5.9	3.5	.4
10	Saline	No	”	6.0	<3.0	<.1
10	Infusion broth	”	”	6.0	<3.0	<.1
20	Water	Yes	30% peptone	6.0	5.0	10
10	”	”	*Idem*	6.0	5.0	10

* See text for details. Original concentrations are given.

† Reciprocal of log PD_{50}.

‡ "Yes" indicates material kept cold throughout drying procedure. "No" indicates material allowed to reach room temperature during drying.

so that the final brain concentration was 10%. Either the reconstituted material underwent a single cycle of rapid freezing to –70°C followed by rapid thawing, or reconstitution was allowed to take place at 4°C for 6 hours. In any one experiment where drying technics were being compared, only one of these methods was used. *Heating of dry MEFl virus.* The material to be heated was dried as 20% brain suspension in 18% peptone (final concentrations). After sealing, the tubes were held at –70°C until they were heated. Control (unheated, dry) material was kept at –70°C until it was reconstituted for titration. Duplicate tubes were heated at 10° increments from 0° to 80°C for 20 minutes. Other duplicate series were heated at 40°C, 50°C, and 60°C for various times. The entire tube was immersed in a water bath whose temperature varied ±1°C. At the end of the holding period it was immediately plunged into dry ice-alcohol and kept at –70°C until it was reconstituted in water and titrated in mice. The contents of duplicate tubes were pooled for titration.

The results of one experiment are included below in which virus was dried at room temperature in summer (about 35°C) and stored immediately thereafter in sealed ampules at 37°C for 70 days. Titrations were carried out immediately after their removal from the lyophilizer, and at 7, 13, and 70 days thereafter. The time elapsing between the disappearance of external ice and water from these tubes and titration of the virus before storage was approximately 2 hours.

Results. Table I shows the titers before and after lyophilization of MEFl virus in various media at room temperature and in the cold. It can be seen that % survival was best when the material, while drying, was kept cold in the presence of peptone or Na thioglycollate with reconstitution in water, or when it was dried in water and then reconstituted in 30% peptone. The remaining media gave intermediate to very poor results when water was used for reconstitution.

Not included in Table I are the results of one experiment in which Type 3 (Leon) virus was dried in the cold as 5% mouse cord in 15% peptone. Before drying, its PD_{50} was $10^{-2.6}$. After desiccation and reconstitution in water, the titer was $10^{-1.6}$.

The results of heating the dry MEFl virus are presented in Fig. 1 and 2. Fig. 1 indicates that holding the preparations for 20 minutes

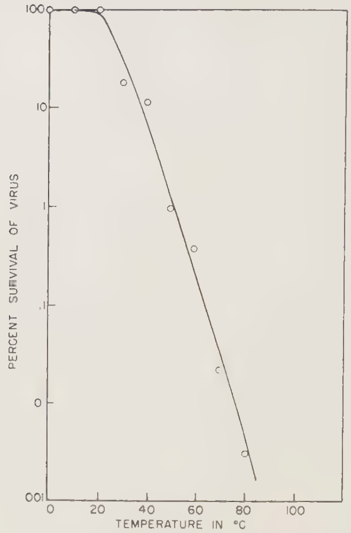

FIG. 1. Heat inactivation of dry MEF1 virus at constant time (20 min.).

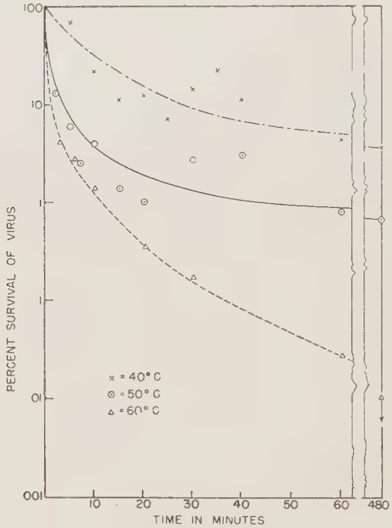

FIG. 2. Inactivation of dry MEF1 virus at 3 temperatures. Arrow at extreme right of the 60°C curve indicates virus survival of less than 0.01%.

at 20°C and below caused no demonstrable activity to be lost. Between 20° and 30°C degradation began and then followed a reasonably straight line when log of % survival is plotted against temperature. When temperature was held constant (Fig. 2) and time varied, the curves derived are no longer exponential. Instead, they tend to flatten out with increasing time. In the single trial in which dry MEFl virus was held at 37°C for 70 days, we assume that, had the material been cold during drying instead of at about 35°C, the control titer would have been comparable with that of other controls, *viz.*, about 10^{-5}, and that the titer, $10^{-3.5}$, obtained just before storage at 37°C represented roughly 3% of the original dry activity. At any rate, after 7 days at 37°C, the titer was $10^{-2.7}$, at 13 days, $10^{-1.8}$, and at the end of 70 days it was $>10^{-0.7}$, 7/7 mice becoming paralyzed and dying when this concentration alone was tested.

Discussion. The results of our experiments indicate that lyophilization of Type 2 and Type 3 poliomyelitis viruses can be accomplished with little loss of infectious activity. This is in contradiction to the current belief that poliomyelitis virus does not survive desiccation(10). If drying is carried out at a low temperature, and if the virus is lyophilized *or* reconstituted in a suitable medium, there is little loss of infectivity. The results further indicate that peptone, Na thioglycollate, and cysteine afford good, or reasonably good, protection for the virus, thus suggesting that mild reducing conditions (as found in --SH compounds) may be necessary to prevent loss of infectivity. This appears to be true even when the temperature is kept low during desiccation. Peptone, containing a variety of amino acids, probably meets the requirement in the same way. Search for the precise mechanism of protection might be undertaken with highly purified virus preparations, but this has not yet been attempted.

The salutary effect of the low temperature during drying is explained by the heat inactivation curve of Fig. 1, for at the usual room temperature, 20°C to 30°C, inactivation begins to appear even at the end of 20 minutes. Thus, when all external ice and moisture have gone from the lyophile tubes and the last traces of water are being removed from within, the entire contents are subjected to room temperature for several hours. During this time a variable amount of heat inactivation can occur.

Because the curves in Fig. 2 are not straight lines, the line drawn in Fig. 1 cannot be taken too seriously. Heating at 40° and 50°C (Fig. 2) brings out the fact that a heat resistant virus population is present in our preparations, for there is little or no difference between the % survival at 60 minutes and 480 minutes in either case. At 60°C, however, this heat resistant segment itself begins to decay, for the curve continues downward although it still does not seem to be a straight line, and at 480 minutes no virus could be detected at 0.01% survival. In addition, the fact that infective virus was still present in the preparation held at 37°C for 70 days speaks for the existence of a heat resistant population. Experiments are in progress to study the heat resistance of MEFl virus and to ascertain whether or not the same inhomogeneity with respect to heat stability applies to the mouse adapted Type 1 and Type 3 viruses.

Although in such a mixed virus population the analysis of the thermal constants is not of precise significance, the figures obtained by assuming that two varieties are present are of interest. The fast inactivating component has a heat of activation of 28,800 calories per mole and an entropy of activation of +21 calories per mole per degree. The slow component has a heat of activation of 20,800 and entropy of –10 in the same units. This last indicates that in the dry condition the slow component is capable of withstanding a wide range of temperatures without becoming inactivated.

The epidemiological implications of these results are obvious if they are applicable to poliomyelitis viruses in general; and the possibility that dry virus may play some part in the dissemination of disease cannot be put aside. If conditions in nature are adequate for the protective drying and/or reconstitution (in the host?) of the dry virus, this becomes more than a possibility. Even though full activity has not been retained in the material dried as outlined above, it has been shown that 10 to 30% of the original activity can be recovered after drying and that, in addition, when exposed to air, a segment of the viral population is quite heat resistant and persists for some time. It remains to be seen, however, if desiccation of natural virus-containing materials such as feces, sewage, saliva, naso-pharyngeal exudates, etc., will yield similar results, and it would be important in this regard to gather information on the degradation of the dry virus during several cycles of drying and reconstitution, ultraviolet inactivation, temperature variation applied to a single dry preparation, and any other conditions that simulate natural climatological events. We have simulated nature in only one respect, *i.e.* the dry virus was heated in the presence of air.

Summary. 1. Data are presented showing that the MEFl (Type 2) baby mouse adapted strain of poliomyelitis virus can be lyophilized with little loss of infectious activity. The same appears to be true of the Leon (Type 3) mouse adapted strain. 2. Heating the dry MEFl virus indicates that, although part of the viral population appears to be relatively heat labile, a measurable proportion of the virus particles is quite resistant to heat. 3. The epidemiological implications of the possible presence of dry poliomyelitis virus in nature are discussed.

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Morphology of Type II Poliomyelitis Virus (MEFl) as Determined by Electron Microscopy.* (21082)

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Previous attempts to determine by electron microscopy the size and shape of particles of human poliomyelitis virus have been equivocal because of uncertainty as to the identity of the virus particle among various objects of similar appearance found in the electron micrographs (1-6). Improved methods of purification applicable to poliomyelitis virus propagated in central nervous system tissue *in vivo,* or in monkey kidney tissue culture, now yield virus concentrates containing at most 2 classes of particles and frequently only one(7,8). Furthermore, the examination of such purified virus concentrates by analytical electron microscopy has resulted in the establishment of a constant ratio of numbers of the particle believed to be the virus to the infectivity titer (8). In a preliminary determination this particle has been found to be approximately spherical and about 28 mμ in diameter, but its successful identification seems to justify at this time a more definitive examination of its shape and size.

Materials and methods. The virus used in this study is a tissue culture MEFl strain received from Dr. Jonas Salk in 1952. It has been passed 3 times in monkey kidney tissue culture in this laboratory, and the third passage has been used as a virus seed pool for the production of liter quantities of tissue culture virus suspension for purification purposes. The method of purification employed was similar to that described by Bachrach and Schwerdt for the purification of Lansing virus from infected cotton rat brains and cords(8). The essential steps of the procedure are: the isoelectric precipitation of virus infectivity from tissue culture fluid at pH 4; the resuspension of the precipitated virus in a one-tenth or one-twentieth volume of 0.94 M NaCl buffered at pH 7.8 with 0.02 M Na_2HPO_4; 2 n-butanol extractions of the molar salt solution extract of the precipitated virus; concentration of the virus from the extracted aqueous phase by one cycle of differential centrifugation in the Spinco, model L, ultracentrifuge with isotonic saline buffered at pH 7.8 as solvent; treatment of the virus concentrate with crystalline ribo- and deoxyribonucleases; and a final concentration and purification of the virus by a second cycle of differential ultracentrifugation. The final preparation usually represents a thousand-fold concentration of the virus from 1 liter of tissue culture fluid. The increase in specific activity of the purified preparation above that of the original tissue culture fluid as estimated by the plaque assay method of McClain and Schwerdt(9) ranges from 200 to 400-fold in 4 purification experiments. Specimens for electron microscopic examination were prepared by normal air-drying and by the freeze-drying technic(10). Prior to preparing an air-dried specimen, an aliquot of the purified virus concentrate was dialyzed against 0.1 M ammonium acetate in order to replace the non-volatile salts of the isotonic saline with a wholly volatile salt solution(11). The virus preparation was then irradiated with ultraviolet light for a sufficient length of time to reduce the infectivity to a level no longer detectable by tissue culture assay. The irradiation reduced the hazard of releasing aerosols of highly infectious material during the manipulations involved in the preparation of specimens, yet did not appear to alter the morphology of the virus particle. Macrodrops of the dialyzed, irradiated virus suspensions were permitted to dry in air at room temperature on Formvar-coated specimen screens. In some instances the virus particles were resuspended on the membrane in a drop of distilled water immediately after the initial drying from the ammonium acetate solution and again allowed to dry. Either before or after the preparation of the virus

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* Aided by a grant from the National Foundation for Infantile Paralysis.

specimen, a dilute suspension of polystyrene latex (PSL) particles was sprayed upon the Formvar membrane. The PSL spheres are uniformly 260 mμ in diameter and serve as a yard stick for the measurement of the diameters of the virus particles and for the determination of the local shadow angle. For the preparation of frozen-dried specimens, aliquots of the virus concentrate were dialyzed against 0.1 M ammonium bicarbonate, irradiated as described above, and sprayed from a high-velocity gun. The spray thus formed consists of micro-droplets of volumes of the order of 10^{-9} ml and these freeze upon and adhere in the frozen state to collodion-coated specimen screens which have been pre-sprayed with PSL particles and precooled to about $-80°$C(10,12). The ice and volatile salt in the frozen droplets were then sublimed in a vacuum at $-50°$C, well below the eutectic temperature of an ice-ammonium bicarbonate mixture. In order to enhance the volatilization of the ammonium bicarbonate the specimen was finally heated to about 60°C for 10 minutes before air was admitted to the freeze-drying unit. The non-volatile constituents remain behind, undisturbed by the surface tension forces which distort individual virus particles in specimens prepared by air-drying. Both the air-dried and frozen-dried specimens were shadowed with uranium before electron microscopic examination. Measurements of virus particle diameters were made by direct examination of the original photographic negative by means of a microscope equipped with an eyepiece micrometer scale.

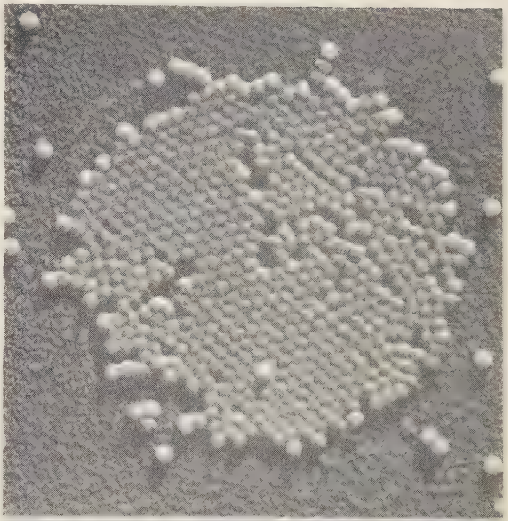

FIG. 1 (top). Poliomyelitis virus (MEFl) air dried from an ammonium acetate suspension. The uniformity of particle size is demonstrated by the existence of regions of close packing. The individual particles are flattened slightly and appear larger than those in the crystalline array. ×74000.

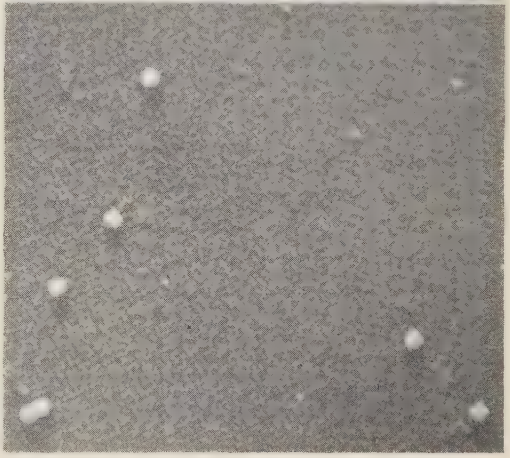

FIG. 2 (bottom). Poliomyelitis virus (MEFl) frozen dried from an ammonium bicarbonate suspension. The particles appear spherical, with no evidence of flattening. They have the same diameter (27 mμ) as those in the packed array in Fig. 1. Shadowed at $\tan^{-1} 0.36$. ×74000.

Results. A typical micrograph of an air-dried specimen of MEFl virus is illustrated in Fig. 1. The 2-dimensional hexagonal arrangement of virus particles observed demonstrates a high degree of uniformity of particle size. Measurement of linear arrays of 5 or more particles along any of the 3 axes of a closely-packed hexagon yields an average particle diameter of 27 mμ. The diameter of individual air-dried virus particles measured perpendicularly to the direction of the shadow length is 31 mμ. However, measurements of their shadow lengths, combined with a knowledge of the local shadow angle, shows them to be flattened by about 20%. Most of the measured difference in diameter between the individual particles and the ones hexagonally packed may be ascribed to the effects of flattening.

Fig. 2 illustrates a frozen-dried specimen of purified virus. The very small, irregularly shaped particles seen over the background are believed to be non-volatile impurities in the ammonium bicarbonate solution, since similar debris is observed in ammonium bicarbonate blanks. The individual virus particles are spherical in shape, a conclusion based upon the appearance of the particles themselves and upon the similarity in the shape of the shadows cast by virus and by PSL particles. The average diameter of the frozen dried virus is 27 mμ, in agreement with the value obtained for hexagonally packed air-dried virus. Careful examination of the shadowed, frozen-dried virus particles has failed to reveal any which exhibits regular angular contours or casts angular shadows, as has been found for tobacco ring-spot virus(13) and for several bacterial viruses(14).

Discussion. Individual particles of MEFl poliomyelitis virus appear to be spherical in frozen-dried preparations and to have the shape of slightly flattened spheres in air-dried mounts. The high uniformity of particle diameter is demonstrated in Fig. 1 where rows formed by packing are seen to be straight over distances including a dozen or so particles. Diameter measurements of individual frozen-dried particles offer further evidence of uniformity in that the variation of the measured diameters is not unequivocally greater than the estimated uncertainty of measurement. These conclusions are in distinct contrast to the results published by Sabin, Hennessen and Warren(6) for a type II virus where the electron micrograph shows a great variation of particle size.

The absolute value of the diameter of the virus particles is subject to several uncertainties, owing to possible artifacts of preparation, the finite resolving power of the electron microscope, the calibration of magnification, and the limitations of the measurement of the photographic images. If we assume that no appreciable shrinkage of individual particles has occurred during the formation of an array like Fig. 1, the average diameter of the particles measured in a row is least susceptible to errors of image formation and measurement. If individual particles are measured, the most reliable diameter is probably secured from the frozen-dried preparations where no sign of surface-tension distortion is seen. Our estimate of the uncertainty in the value given for the particle diameter is ±2 mμ.

Summary. The particles of the MEFl poliomyelitis virus, grown in monkey-kidney tissue culture, and subsequently purified by chemical and physical means, are spherical in shape and highly uniform in diameter. The diameter of individual particles, prepared by normal air-drying from a 0.1 M ammonium acetate suspension, is found to be 31 mμ. The diameter of particles packed in a crystalline array, or frozen-dried from a 0.1 M ammonium bicarbonate suspension, is found to be 27 mμ. It is believed that the latter diameter is the more significant and reliable figure.

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A Turbidimetric Assay for Tyrosinase Activity in Frozen Sections of Mouse Skin.* (21083)

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Although the incubation of mammalian skin sections in dopa(1-3) or in tyrosine(4,5) has proved useful in localizing tyrosinase activity, attempts to measure the activity of these preparations have been hampered by the lack of sensitive and objective methods for determining the amount of newly formed dark pigment. Previous estimations of tyrosinase or dopa oxidase activity by visual grading of hair bulbs have served to indicate order of effect as a function of genotype(2,3) or treatment(6). However, a linear scale of visual grades might actually represent much larger changes, involving an exponential relationship, in amounts of pigment(7). Moreover, the visual grading method involves shifting standards for comparing enzyme activities in differently colored genotypes. For these reasons it was considered desirable to develop a sensitive method for assaying tyrosinase activity objectively and on an absolute scale of turbidimetric measurements.

Materials and methods. The slow, partial breakdown of wool keratin in warm, dilute alkaline sulfide(8) and the dispersion of melanin in alkaline media(7,9-12) suggested the possibility that hot concentrated alkaline sulfide might be a suitable dispersion medium for both melanin and keratin. We therefore used a solution molar with respect to both KOH and $Na_2S \cdot 9H_2O$. Hot KOH alone was much less effective in breaking down the skin, although it has been reported to be satisfactory for dissolving human hair keratin(12). Three hundred *frozen sections,* 30 μ thick, from the dorsal skins of two 7-day-old intense brown mouse littermates (genotype bb) were incubated at 38°C for 8 hours in 1½ ml M/10 phosphate buffer, pH 6.8, or in the same volume of buffer containing either L-tyrosine or L-dopa at a concentration of 0.5 mg/ml. Following incubation, the sections were prepared for histological examination as previously described(5). After clearing in toluol, several sections were removed for histological study and visual grading of hair bulbs, while the remaining sections were reserved for turbidimetric tests. Sections used in the latter tests were placed on filter paper to accelerate evaporation of the toluol, and 10 mg samples of dry sections were then transferred to Klett colorimeter tubes. To each of the tubes was added a 5 ml aliquot of dispersion medium. The tubes were placed in a boiling water bath and their contents were stirred occasionally. Within 15 minutes the keratin and pigment underwent dispersion to give brown suspensions. The breakdown of heavily pigmented sections resulted in incomplete dispersion; small, dark, slowly settling, particles were visible. Vigorous agitation of Klett tube contents was therefore necessary before each turbidimetric reading in a Klett-Summerson photoelectric colorimeter (with No. 42 blue filter). The dispersion medium was used for the zero setting of the instrument, and readings for a given suspension were obtained four times in order to determine the variability of the measurements. The turbidimetric stability of the suspensions was checked by storing them overnight in a refrigerator (5°C) and obtaining additional readings on the following day. *Visual gradings* of hair

TABLE I. Comparison of Pigmentary Intensities of Incubated Frozen Skin Sections as Measured by Visual Gradings or Turbidimetric Readings.

Incubation medium	Visual gradings No. bulbs graded	Visual gradings Mean grade	Turbidimetric readings (Klett units)
Buffer	602	3.26 ± .03	295 ± 2.5
Tyrosine	381	3.52 ± .03	489 ± 5.8
Dopa	411	3.88 ± .03	773 ± 6.2

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* Work supported in part by a research grant from the National Science Foundation and by an institutional research grant from the American Cancer Society, administered by the committee on Atypical Growth, Yale University.

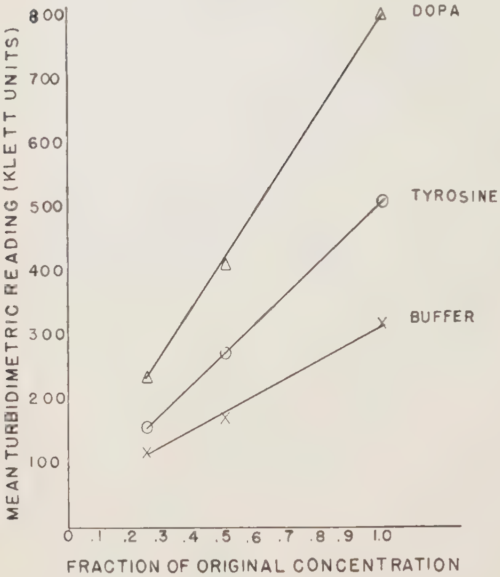

FIG. 1. Adherence of skin and melanin suspensions to Beer's Law as indicated by straight line relationships between turbidimetric readings and degrees of dilution. Only incubation media are indicated.

bulb pigmentation were obtained at low power magnification by one of us (T.A.S.). A scale of 5 grades was adopted to encompass the range from a light brown stippled appearance (grade 1) to a solid black hair bulb (grade 5). Fractional grades were not assigned to any hair bulbs.

Results. Measurements of tyrosinase and dopa oxidase activities by both methods described above are shown in Table I. Mean values of visual grades or turbidimetric readings are followed by their standard errors. The following points are noteworthy: 1) Both methods for determining pigmentary intensity agree as to order of effect. In both cases the differences between mean values are significant at the 1% level. 2) A fractional change in average visual grade corresponds to a turbidimetric change of several hundred Klett units. Thus the latter method is far more sensitive than the former.

The suspensions follow Beer's Law; the relationships between Klett readings (proportional to optical densities) and degrees of dilution appear to be linear. These relationships are shown in Fig. 1. Both medium and suspensions had been stored overnight at 5°C prior to diluting the suspensions and obtaining Klett readings.

Despite the sensitivity of the turbidimetric method it still suffers from 2 shortcomings: 1) The dispersion medium appears to be unstable; a small number of dark particles appear after overnight storage at 5°C. 2) The skin and pigment suspensions showed slight darkening, approximately 20 Klett units, after overnight storage at 5°C, but with readings taken after the suspensions had been warmed to room temperature.

Conclusion. The new turbidimetric method for assaying tyrosinase activity reported here represents a marked improvement over the previously used visual grading method, not only in objectivity and in greatly increased sensitivity, but also in making possible the use of an absolute scale. While immediately useful for determining order of effect, this method might also be sufficiently improved to permit comparison of amounts of naturally occurring pigment in various skin and hair samples.

Summary. A new sensitive and objective method for the turbidimetric assay of tyrosinase activity in frozen skin sections is described. The procedure involves the dispersion of both melanin and skin and hair keratin, from previously incubated sections, in hot molar Na_2S and KOH. Turbidimetric readings are then obtained on the absolute scale of the Klett-Summerson photoelectric colorimeter. This method is immediately useful for determining order of effect, and, if sufficiently improved, might also be useful for comparing amounts of naturally occurring pigment in different skin and hair samples.

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Anticoagulant and Other Pharmacologic Effects of Sulfated Chitin in Animals. (21084)

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The sulfation of various polysaccharides to produce compounds having anticoagulation properties was first described by Bergstrom (1). He reported on the characteristics of the sulfated products of cellulose, starch, glycogen, pectin, chitin and chondroitin sulfuric acid. Most of his investigations were conducted *in vitro,* but later workers, including Piper(2) and others, extended much of the study to *in vivo* testing. A side reaction frequently observed was the agglutination of the thrombocytes. This, coupled with other toxic symptoms, led to the conclusion that the compounds were not suitable for clinical application. Sorenson and Wright(3) described a polysulfuric acid ester of polyanhydromannuronic acid which was tried clinically. Field *et al.* (4) tested the sodium salt or sulfated polygalacturonic acid methyl ester methyl glycoside, and Walton(5) reported on the biological behavior of sulfated dextran. In all instances there was evidence of potential side reactions or toxic phenomena.

Preliminary tests with the sulfuric acid ester of chitin were sufficiently promising to justify extensive studies on the biological behavior of this material. The properties and toxicity of various preparations are herein reported.

Materials and methods. Samples with various average molecular weights were produced by Cushing *et al.*(6), who also described the material as follows: "The product can be defined as the sodium salt of the sulfuric acid ester of poly (1-4) N acetylglucosamine. The sulfur assays (13.5 to 14.5%) showed the degree of sulfation to be somewhat below the maximum theoretical sulfur content of 15.7% for a product having two sulfate groups on each acetylglucosamine residue. The nitrogen assays ranged from 2.3 to 3.2%, the variation being due chiefly to small amounts of residual triethylamine (as a salt on sulfate) used for neutralization of the sulfation mixture. Curiously, the elimination of the residual triethylamine did not significantly decrease the toxicity of the product. All molecular sizes are represented according to the usual frequency distribution curve except that molecules below 5000 mol wt are mostly eliminated during dialysis or repeated alcohol precipitation. In a product having an average molecular weight of 14000, the amount of high molecular weight material (above 17000) is less than 0.5% of the total. In the selected material, relative viscosities (R.V.) in 1% aqueous solution were remarkably uniform, with 1.3 R.V. as the preferred product, and a maximal permissible range of 1.25 to 1.35. The *in vitro* potency varied between 27 and 35 I.U./mg(7)."

Injections were almost exclusively intravenous. Unless high doses were required, sulfated chitin and heparin were used as 1% aqueous solutions. Blood samples for all tests were withdrawn through silicone-treated needles, either into siliconed syringes for transfer to siliconed, chilled glassware or directly into capillary tubes thrust into the needle hub. Coagulation times were determined by the capillary tube method(8). Prothrombin determinations were made by the Quick one-step method. Platelets were counted directly from samples diluted with 3.8% sodium citrate, as described by Quick *et al.*(9). Acute intravenous toxicity was determined in mice. Subacute intravenous toxicity was observed

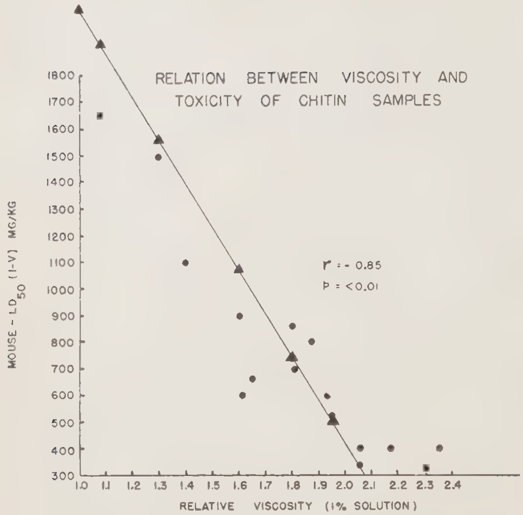

FIG. 1. Triangles = calculated points for regression line. Circles = actual values.

in rabbits, dogs and monkeys with selected preparations.

Results. The *in vitro* potencies of samples having different average molecular weights were assayed by the U.S.P. heparin technic. The acute toxicity in mice was proportional to the average molecular weight (estimated by the R.V. of a 1% aqueous solution.) Fig. 1 shows the LD_{50} of each sample plotted against its relative viscosity. The values closely approximate the calculated regression line, and the correlation is statistically significant (P 0.01). The duration of action was roughly proportional to the R.V.

In contrast, there was no relationship between *in vitro* anticoagulant potency and viscosity, nor did the *in vivo* results show good correlation with *in vitro* values, or provide an indication of duration of action. Therefore, assays in rabbits were chosen as a more reliable criterion of possible usefulness.

Low molecular weight samples had much shorter duration for a given dose; for example, a sample of R.V. 1.1 had a duration of action of less than 30 minutes at a dose of 10 mg/kg (see below and Fig. 2 for data on 1.3 R.V. material). Higher molecular weights were more toxic, as shown in Fig. 1. Finally, material having a R.V. of 1.3 (as described under Materials) was chosen for more extensive study. This represented a compromise between toxicity and duration of action. By osmometric and light scattering methods the average molecular weight of this sample was determined to be between 13000 and 15000. The LD_{50}, in mice calculated by the method of Miller and Tainter(10), was found to be 1.5 g/kg.

Blood samples were taken from dogs and rabbits before, and 5, 15, 30 and 60 minutes after the injection of the compound, or until the coagulation time had returned to or below 3 times the normal coagulation time, which was arbitrarily defined as a limit of "significant drug effect". In rabbits, 10 mg/kg of sulfated chitin had a potency approximately equal to 2.0 mg/kg of heparin, while 5 mg/kg of sulfated chitin was appreciably more potent than 1.0 mg/kg of heparin. With equipotent doses, the duration of "significant drug effect" was 2 to 3 times longer with sulfated chitin. In dogs, 1.5 mg/kg of heparin and 5 mg/kg of sulfated chitin were approximately equal in potency and duration. Platelet counts were performed on the same blood samples. The results in dogs and rabbits were in agreement with the reports of earlier workers. There was a moderate reduction (40-60%) in the number of circulating platelets, but the magnitude of change was no greater, though the duration was somewhat longer than that obtained with equipotent doses of heparin. However, as also reported by others for heparin, the platelet count again reached control levels with or before the return of normal coagulation time. No significant influence of sulfated chitin, in doses up to 10 mg/kg, upon the blood prothrombin level was noted. The anticoagulant effect can be neutralized by protamine sulfate.

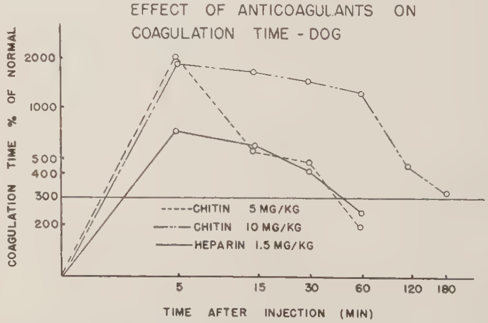

FIG. 2. Horizontal line at 300% of normal coagulation time represents arbitrary lower limit of "significant drug effect."

These findings strengthen the assumption that its physiological action is similar to that of heparin.

Rapid intravenous injection into anesthetized cats or dogs of as much as 50 mg/kg produced no change in blood pressure other than the effect of volume of fluid given. Attempts to sensitize animals with sulfated chitin by methods which detect weak antigens [Roth *et al.*(11)] failed to demonstrate any antigenic potentialities.

Four dogs were given 10 mg/kg daily of a higher molecular weight sample having a R.V. of 1.95. In all 4 animals, bloody diarrhea and vomiting occurred after 6 to 8 days of treatment. In those sacrificed for histological study, acute hemorrhagic changes were confined to the intestine. All other tissues showed no significant pathological changes.

Daily intravenous administration to dogs of 10 mg/kg of 1.3 R.V. sulfated chitin produced no significant symptoms for the first week. After about 10 days some lethargy and slight ataxia appeared but no other symptoms were noted, and animals sacrificed showed no gross or microscopic abnormalities. At 20 mg/kg per day, the animals showed some loss of weight, and after 8 days ataxia and weakness appeared. Death occurred at 9 and 10 days, but the animals were not seen in time to justify autopsy. No diarrhea occurred with either dose level. These untoward effects were entirely absent in rabbits and monkeys. Daily doses of 20 mg/kg for 3 weeks produced no detectable symptoms in either species. Some rabbits received 50 mg/kg/day for 2 weeks without untoward effects, while others tolerated 100 mg/kg/day for a few days. Death ultimately resulted from direct hemorrhage from injection sites, reopened after return to their cages. Hematological studies of animals during chronic administration showed no significant departure from normal values for red or white cell count, differential count or hemoglobin.

Attempts were made to eliminate unknown toxic factors and to improve purity by modifying the chemical process. The mortality data obtained with numerous samples on a total of 4044 mice (at least 30 per sample) provided LD_{50} values ranging between 1.25 and 1.8 g/kg which represented no encouraging improvement over earlier material.

Discussion. Survey of recent literature disclosed several reports which express doubts as to the safety of various sulfated polysaccharides. Astrup(12) observed that citration of blood appears to stabilize human platelets. Therefore, if citrate is used for any observations on the effects of anticoagulants on platelets, negative results may be misinterpreted. He also implied that any synthetic anticoagulants of the sulfated polysaccharide type might be expected to agglutinate thrombocytes and would be considered undesirable for clinical use. Other reports (Pratt(13); Fischer *et al.*(14); Hirschboeck *et al.*(15)) described the occurrence of alopecia following the use of synthetic anticoagulants, and Wright(16,17) reported many other side effects. In our results on dogs, it was first thought that the effects were possibly a species idiosyncrasy similar to that observed with polyvinylpyrollidone. However, Hirschboeck (15) describes the occurrence of a bloody diarrhea in human patients treated with another sulfated polysaccharide. This information indicates the need for more information on the basic mechanisms involved, and for improvement in the synthetic approach, before a completely safe heparin substitute can be assured.

Summary. The anticoagulant effects of a sulfated polysaccharide, the sodium salt of the sulfuric acid ester of chitin, have been described. It has an *in vivo* potency ¼ to ⅕ that of heparin activity. Duration of action and toxicity are both directly related to average molecular weight. The occurrence of toxic side reactions in the dog are similar to some of those reported in the human for another sulfated polysaccharide. Limitations of potential clinical usefulness are discussed.

The authors express appreciation to R. A. Hendricks and B. B. Morphis for valuable technical assistance and to R. T. Olsen and R. A. Rieker for the *in vitro* potency assays.

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Intracellular Pattern of Nucleic Acid in Virus Infection.* (21085)

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(Introduced by Thomas Francis, Jr.)

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Upon infection with herpes virus, the heart and liver of chick embryos reveal an enlargement which results from an increased rate of cellular proliferation. This is paralleled by a markedly increased rate of nucleic acid synthesis such that the total nucleic acid of the liver may be 70% greater than normal(1). While these first observations were concerned with differences in the whole organ which were a result of the increased mass of the liver, the present paper deals with observations at the cellular level.

The amount of nucleic acid per cell has been estimated in normal and viral infected tissues. Nuclei isolated from herpetic liver have been analyzed for the nucleic acids, and data concerning the intracellular distribution of deoxyribonucleic acid and ribosenucleic acid have been obtained. Parallel observations have been made of the nucleic acid pattern of the Ehrlich ascites tumor infected with influenza virus.

Methods and materials. Virus. The herpes virus used was the Armstrong 1166 strain. It had undergone 54 passages in mouse brain and was adapted to the embryonate egg by 40 passages on the chorionic membrane(2). The WS strain of Type A influenza virus was used in the studies with the Ehrlich mouse carcinoma. This virus was maintained for several years by continuous passage in tissue culture and since has undergone 102 passages in mouse brain. It will multiply both in the brain and lungs of mice. After adaptation to the Ehrlich ascites tumor, it has been passed serially in that tissue 40 times(3). *Tissue.* The Ehrlich mouse carcinoma was adapted to ascitic form by Dr. G. A. LePage and in this laboratory has undergone 60 passages in the ascitic form in Swiss white mice (Webster strain). *Fractionation.* A 20% homogenate of liver was prepared in 0.25 M sucrose containing .0018 M $CaCl_2$. The disruption of the tissue was achieved with the Potter-Elvehjem glass homogenizer. The resulting preparation was diluted to 4% and fractionated essentially by the procedure of Hogeboom and Schneider(4). A nuclear and a cytoplasmic fraction were obtained. The latter contained the soluble proteins as well as the mitochondria and microsomes. All operations were performed at temperatures

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TABLE I. Nucleic Acid Content per Cell of Normal and Virus Infected Chick Liver.

	No. nuclei* $\times 10^{-7}$	NA-P $\times 10^{7}$/cell‡	DNA-P $\times 10^{7}$/cell‡	RNA-P $\times 10^{7}$/cell‡	$\frac{\text{DNA-P}}{\text{RNA-P}}$
Normal†	33.4 ± 1.3	16.8 ± .8	4.4 ± .5	12.4 ± .8	.35
Infected†	34.2 ± 1.5	16.7 ± 1.2	4.3 ± .6	12.4 ± .7	.35

* No. of nuclei in 400 mg moist liver.

† Each value (± stand. dev.) is the average of 7 determinations.

‡ Calculated on assumption that there is 1 cell per nucleus and expressed as μg of nucleic acid phosphorus per cell.

between 4° and 0°C. *Nuclei counts.* To determine the number of nuclei per unit weight of liver, suitable dilutions of a 20% homogenate of tissue were made in 1% citric acid. The nuclei hardened by citric acid were then counted in a hemocytometer. *Nucleic acids.* Deoxyribonucleic acid (DNA) and ribosenucleic acid (RNA) were determined by the procedure of Schneider(5). The RNA was calculated from the difference between the total nucleic acid and the DNA. Phosphate was estimated by the modified Fiske and Subbarow method(6).

Results. Nucleic acid of normal and herpes infected livers. Inoculation of an adapted strain of herpes simplex virus on the chorionic membrane of an egg is followed by viral infection not only of the membrane but also of the liver, heart, and brain of the embryo. Because of the size of the organ, the extensive lesions obtained, and the ease of cellular fractionation, the liver was chosen as a suitable material for these studies. Eighteen 12-day-old eggs were inoculated via the chorionic membrane with 0.2 ml of herpes virus of titer $10^{-5.5}$ ID_{50} for eggs. After incubation for 3 days at 38°C, the eggs were opened and the livers of the embryos were removed. In all cases the infection had spread to the embryo and there was evidence of extensive hepatic lesions. A control group of eggs which had not been infected was treated in like fashion. From the harvested tissues, 20% homogenates were prepared in 0.25 M sucrose which contained .0018 M $CaCl_2$. Suitable dilutions of the 20% homogenates were made in the solution of calcium and sucrose used originally to prepare the homogenates. For each procedure 2 aliquots of the diluted homogenate were taken in order to obtain duplicate values. The number of nuclei per ml of homogenate was determined. Each gram of moist embryonic liver contains approximately 80 x 10^7 nuclei. The count per unit weight of moist tissue is slightly but consistently greater in the infected tissue. The results are in agreement with previous determinations(1). With this value for the number of nuclei and the analysis of other aliquots for nucleic acid, the amounts of DNA and RNA per cell were calculated.

The data from 7 similar experiments were combined and recorded in Table I. On the average, these cells contained 16.8 x 10^{-7} μg of nucleic acid phosphorus which is distributed between RNA and DNA in 3:1 ratio. It is clear from the data of Table I that on the basis of the nucleic acid analyses the infected tissue is undistinguishable from normal liver. Within the limitations of the method, the total nucleic acid and the ratio of DNA to RNA are found to be unchanged by the infection which has produced extensive and visible alterations in the liver.

Intracellular distribution of nucleic acids. When infected tissue is subjected to controlled cellular disruption the major part of the herpes virus is found in the cytoplasmic fractions of the cell(2,7). The detection of viral nucleic acid in the cytoplasmic fractions by the methods employed here would not be expected. However, alteration in the intracellular distribution of nucleic acids might be anticipated from other considerations. If herpes virus is formed at some extranuclear site as postulated, the synthesis of viral nucleic acid in the cytoplasm or the transfer of nucleic acid from the nucleus to cytoplasm might be in excess of that found ultimately in the viral body. Since DNA is confined to the nucleus of the normal cell, and the other cellular fractions are essentially free of DNA,

TABLE II. Intracellular Distribution of Nucleic Acids in Normal and Virus Infected Chick Liver.

	Normal		Infected	
Preparation*	DNA-P mg†	RNA-P mg†	DNA-P mg†	RNA-P mg†
Whole homogenate	.15	.40	.14	.37
Nuclei	.14	.26	.13	.24
Supernate‡	.01	.15	.01	.14

* Each preparation is equivalent to 400 mg moist liver.

† Nucleic acid phosphorus in mg/preparation.

‡ Fraction contains soluble cytoplasmic elements, mitochondria and microsomes.

the nuclear and cytoplasmic fractions of normal and infected tissues were separated and analyzed for both nucleic acids. The resulting data are recorded in Table II. Approximately one-third of the total RNA is found in the nucleus of the embryonic chick liver. Despite the nuclear aberrations seen in infected cells, the amount of nuclear DNA and RNA is unaltered, nor does one find abnormal amounts of contaminating DNA in the extranuclear fractions. The evidence of these experiments suggests that the development of herpes virus proceeds without any gross alterations in the amount of nucleic acid per cell, in its distribution between RNA and DNA, or in the distribution of nucleic acids within the major cellular fractions.

Nucleic acid of control and infected tumor cells. Since the cells of herpetic liver contain on the average 36% less mitochondria than normal liver cells, a minimum value of 36% can be set for the fraction of cells involved in an infected tissue. If, as one might suppose, the destruction of mitochondria in an infected cell is less than 100%, then the number of infected cells in a tissue may be considerably greater than 36%(3). However, comparisons of the type made here are always between a mixture of normal and infected cells on the one hand and normal cells on the other. Because it seemed that any metabolic alteration would be more readily demonstrated with a system containing a high degree of cellular infection, the host-virus system composed of influenza virus and the Ehrlich mouse ascites tumor was included in the study. Mice inoculated 7 days previously with 2 x 10^6 ascites tumor cells were infected with 0.1 ml of a preparation of adapted influenza virus. This inoculum had a titer in eggs of $10^{-7.5}$ ID_{50}. After 22 to 28 hours when the titer of the peritoneal fluid had reached a maximum, fluids containing cells were removed from infected and control groups of mice. The cells were removed from the fluid, washed and counted. Separate aliquots were analyzed for RNA and DNA. The amounts of these nucleic acids per cell were calculated and the resulting values are found in Table III. The proportions of DNA to RNA are much higher in these cells than in those of the chick liver. However, the comparison of normal and infected tissue is completely consistent with that found in Table I. In other experiments tumor tissue was removed from mice which were infected with influenza virus 42 hours previously and in which the ascites cells had begun to aggregate and form solid tumors. To obtain comparable control material, a few uninfected mice were chosen which had solid tumors in the peritoneal cavity. The amounts of nucleic acids per gram of moist tissue were determined in each case and the resulting values presented in Table III. Again it will be noted that the pattern and distribution of nucleic acid between RNA and DNA are the same in control and infected tissue. These data with influenza virus like those from the herpetic infection indicate no measurable change in the amount of RNA or DNA per cell that is induced by infection with influenza virus.

Discussion. Subsequent to infection of the chick liver with herpes virus, several changes are detectable There is a loss of mitochondrial material and a relative change in the abilities of the tissue to oxidize succinic and α-ketoglutaric acids(1). Despite these alterations, the static pattern of the nucleic acids seems to remain fixed as the transformation of the normal cell to a viral producer is accomplished (Table II). The rather large increase (70%) in the rate of nucleic acid synthesis by the liver, as reported previously, must result entirely from the accelerated cellular proliferation(1). The increased production of liver nucleic acid is paralleled by an increased number of cells which on the average contain the normal amount of nucleic acid.

No unnatural amount of nuclear material

TABLE III. Nucleic Acid Content of Control and Influenza Infected Ascites Tumor Cells.

Tissue	Cells/ml × 10^{-6}	NA-P* × 10^6/cell	DNA-P* × 10^6/cell	RNA-P* × 10^6/cell	$\frac{\text{DNA-P}}{\text{RNA-P}}$
Control†	19.7 ± 3.5	5.5 ± .2	2.4 ± .0	3.1 ± .2	.79
Infected‡ 22-28 hr	21.8 ± 4.6	5.3 ± .4	2.2 ± .2	3.2 ± .2	.69

Tissue	—	NA-P mg/g§	DNA-P mg/g§	RNA-P mg/g§	$\frac{\text{DNA-P}}{\text{RNA-P}}$
Solid tumor control	—	.97	.45	.52	.87
Solid tumor infected 42 hr	—	1.00	.47	.52	.90

* Nucleic acid phosphorus expressed in μg per cell.

† Each value (± stand. dev.) is average of 2 determinations.

‡ ” ” ” ” ” ” 3 ”

§ mg of nucleic acid phosphorus per g of moist tissue.

(DNA) is found with the cytoplasm, as might be expected if fragmentation of the nucleus were a major feature of infection. These data, as well as those of other recent studies(8), support the concept of a stable structural integrity including the external cellular membrane which is maintained throughout the period of viral development and liberation. Apparently the subtle metabolic injuries which must occur are manifest only later in a general dissolution of cellular structure.

It should also be noted that, while there are aberrations in the nucleus (inclusion bodies) which are Feulgen staining in infected cells, this abnormality was not detectable by the chemical analyses for DNA and RNA (Tables I and II). Quite possibly this finding is an inevitable result of the limitations of the experiments which lie in the grossness of the methods, and measurements of the dynamic state of the nucleic acids might be much more rewarding. However, as yet, there is no clear evidence that the nuclear bodies contain significant amounts of infectious virus, and the nature and origin of these structural changes remain unexplained. That the nuclear aberrations appear close upon the initiation of infection is an extremely interesting observation (9), but does not allow one to decide whether they are developmental forms of the virus or secondary cellular injury(2).

Summary. The nucleic acid content per cell of chick livers was found to be unaltered as a consequence of infection with herpes virus. The distribution of the nucleic acid between ribosenucleic acid and deoxyribonucleic acid is the same in normal and infected tissues. The intracellular distribution of nucleic acids was determined and the data suggest a remarkable stability of the nucleus of the infected cell. Within the extent of the investigation, the nucleic acid pattern of the Ehrlich ascites tumor after infection with influenza virus was found to parallel that of the herpetic liver cell.

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Influence of 19-Nortestosterone Cyclopentylpropionate on Urinary Nitrogen of Castrate Male Rat. (21086)

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Since the first demonstration of the nitrogen-sparing metabolic action of androgenic steroids(1), there has been much research to determine the activities of many androgens with respect to protein anabolism. High anabolic activity with relatively low androgenic activity has been reported for 17α-methyl androst-5-ene-3β,17β-diol(2,3) 6β-hydroxy-3, 5-cycloandrostan-17-one(4), 17β-hydroxy androstan-3-one(5), 17α-methyl androstan-17β-ol(6), 17β-hydroxy estr-5(10)-en-3-one, 19-nortestosterone(7), and three esters of the latter compound(8). "Anabolic" activity has been attributed to many compounds based on indirect measures—increase in weight of kidney(9), levator ani(10) or temporal muscles (11), increase in body weight, etc. Although these criteria are of great value in preliminary work or for precise quantitative data, we believe that direct evidence of nitrogen retention in the whole animal is the most nearly indisputable evidence for protein anabolic effect. The nitrogen balance of the rat has been used as a test for anabolic activity by Kochakian and his co-workers(12) and by Kassenaar, van Bekkum, and Querido(4). Although such experiments are frequently not adaptable to precise quantitative interpretation, they do indicate order of magnitude of potency.

This report deals with limited studies of the effect of NTCP (19-nortestosterone cyclopentylpropionate*) on the urinary N of the castrate rat in comparison with TP (testosterone propionate), 19-nortestosterone, MAD (17α-methyl androst-5-ene-3β, 17β-diol), and androstanolone (17β-hydroxy androstan-3-one).

Methods. The rats used were large males from the Upjohn colony castrated for long periods of time before treatment. They were housed in individual metabolism cages and force-fed twice daily with the Ingle medium-carbohydrate diet† 28 g/rat/day (390 mg nitrogen). This diet caused weight gain and in the earlier experiments the animals were all in positive N balance. In the later experiments growth had virtually stopped and the rats were in balance. Water was given *ad libitum*. Twenty-four hour urine specimens were collected 3 times weekly and analyzed for total N(13). Results were calculated as mg N/24 hours. The steroids were given by subcutaneous injection of .1 ml cottonseed oil solution. The details presented above are common to all experiments; other conditions which varied from one experiment to another are described below.

Results. Comparison of effects of NTCP and TP. Rats were castrated at 25 days of age and kept, untreated, for 67 days before beginning force-feeding and transfer to metabolism cages. At the beginning of force-feeding, the average weight was 300 g. The transition from stock diet to controlled intake of liquid diet was accomplished by beginning with 10 ml/rat/day and increasing the amount 2 ml/day to a level of 26 ml/rat/day. Steroid treatment was begun 24 days after the maximum feeding level was attained, when the rats weighed an average 350 g. Weight gain con-

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* A paper describing preparation, physical and chemical properties of this compound is in press (*J. Am. Chem. Soc.*): Babcock, J. C., R. A. Donia, B. A. Johnson, and A. C. Ott.

† Composition of this diet is as follows:

Cellu Flour	180 g
Salt Mixture No. 2 Nutritional Biochemicals	120
Brewer's yeast	300
Casein	480
Starch	600
Dextrin	570
Sucrose	600
Water	3330 cc
Corn oil	570
Cod liver oil	30
Wheat germ oil	30
Vit. K, 0.5% sol. in cottonseed oil	30

tinued so that after 30 days of steroid treatment mean body weight was 410 g and 3 weeks after cessation of treatment was 460 g. The weight increase was the result of the restricted activity and more-than-adequate diet combining to cause the rats to become obese. In addition to fat there was deposition of protein, as the rats were in positive N balance at all times. Two groups of rats were treated with TP in doses of .25 mg or 1 mg and 2 groups with NTCP in doses of .5 mg or 2 mg/rat/day for 30 days. Fig. 1 and 2 show the N excretion and weight changes of the 4 groups. The base-line for the graphs is the mean pre-injection N excretion. The lines drawn through the body weight curves are projections of the pre-treatment growth rate, not intended to represent the expected growth for an untreated animal, but as base-lines for evaluating changes in weight during treatment. Both doses of both compounds caused substantial N retention initially. After a time N excretion increased in spite of continued treatment, continuing to rise at about the same rate after cessation of treatment. The rate of increased N excretion after the wearing off of the steroid effect was the same as that of untreated control animals.

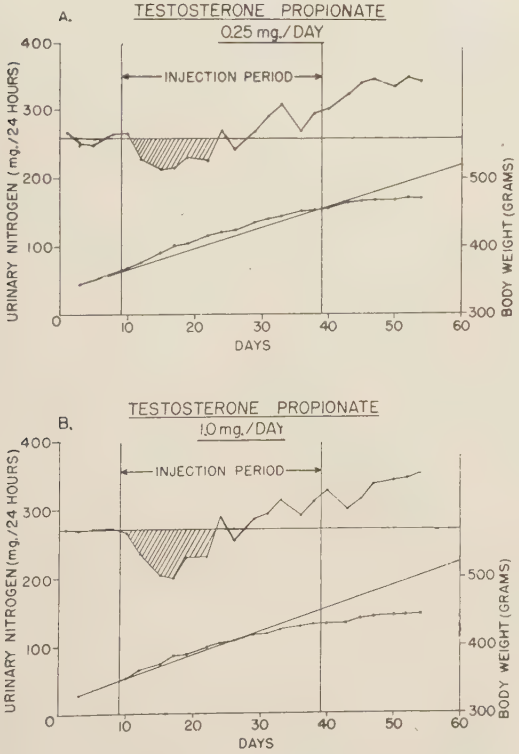

FIG. 1. Urinary total nitrogen and body weight changes of rats treated for 30 days with .25 and 1 mg TP. Shaded areas indicate significant nitrogen retention during injection period.

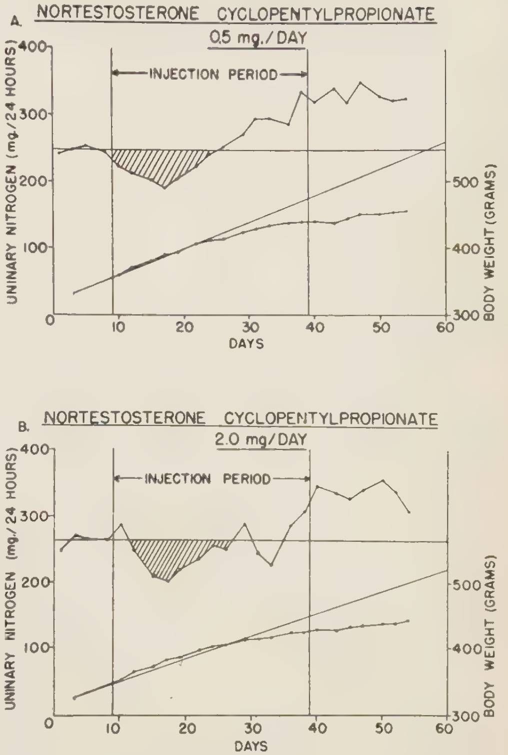

FIG. 2. Urinary total nitrogen and body weight changes of rats treated for 30 days with .5 and 2 mg NTCP. Shaded areas indicate uninterrupted nitrogen retention during injection period.

Three indices of potency have been used in evaluation of these data: The "greatest daily retention" is the difference between the lowest daily N value after beginning of treatment and the pre-injection average. The "total N retention" is the summation of the differences between pre-injection excretion and the daily values during the retention period. The values of the days on which N was not determined are estimated as being the mean of the experimentally determined values before and after. The "number of days in the retention period" is the period between the last day on which N was equal to or higher than the pre-injection average and the last day

TABLE I. Summary of Nitrogen Excretion of Rats Treated with Varying Doses of Testosterone Propionate and 19-Nortestosterone Cyclopentylpropionate.

	Testosterone propionate		Nortestosterone cyclopentylpropionate	
	0.25 mg/day	1 mg/day	0.5 mg/day	2 mg/day
No. of rats	5	5	4	5
Total N retained, mg	398	604	507	483
No. of days in retention period	16	20	16	16
Greatest single daily retention, mg	44	73	54	60

before two consecutive values equal to or higher than the pre-injection average were obtained.

Table I lists the values of these indices in this experiment. NTCP at .5 mg/day produced a greater response than TP at .25 mg, but a lesser response than TP at 1 mg, indicating that NTCP is more than half but less than twice as potent as TP. NTCP produced essentially the same response at 2 mg as at .5 mg. Until some explanation is found for this lack of proportionality between dose and response, it is not possible to derive a firm potency ratio for the two compounds. From examination of the data it appears reasonable to conclude that the N-retaining potencies of NTCP and TP are of the same order of magnitude. The weight change curves reveal that both compounds produced slight acceleration of weight gain (Fig. 1 and 2). This is consistent with experience of other workers(14) with hormones causing retention of N.

Short courses of treatment with NTCP, MAD, and androstanolone. The rats were the same animals used in the experiment described above. The beginning of treatment in this experiment was 67 days after the last injection in the previous experiment, a period presumed

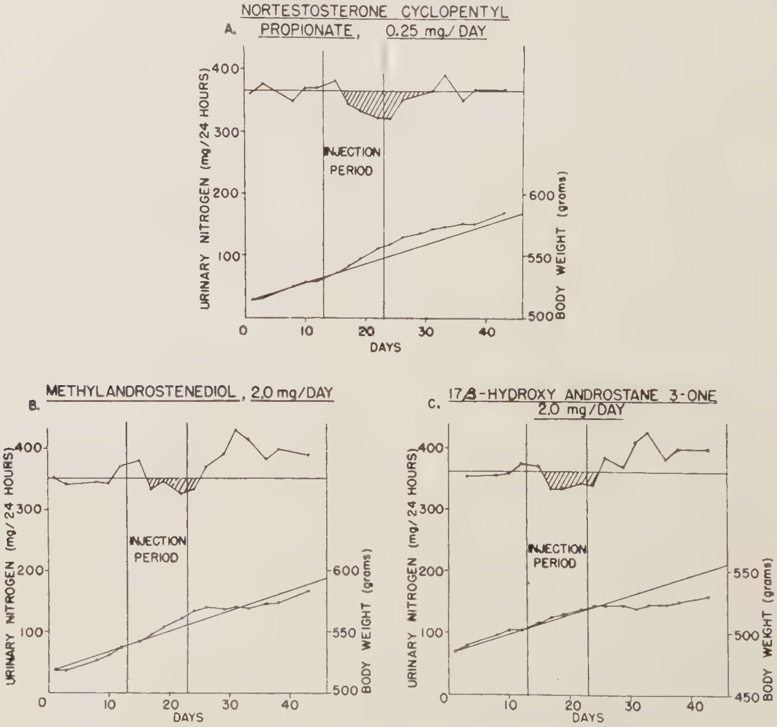

FIG. 3. Urinary total nitrogen and body weight changes of rats treated for 10 days with .25 mg NTCP, 2 mg methylandrostenediol, or 2 mg androstanolone. Shaded areas indicate nitrogen excretion less than that of fore-period.

TABLE II. Nitrogen Excretion of Rats Treated with Nortestosterone Cyclopentylpropionate, Methylandrostenediol, and Androstanolone.

	Nortestosterone cyclopentylpropionate, 0.25 mg/day	Methylandrostenediol, 2 mg/day	Androstanolone, 2 mg/day
Total N retained, mg	342	128	196
No. of days in retention period	14	9	9
Greatest single daily retention, mg	43	24	27

sufficient to permit recovery from the previous treatment. At the beginning of the injection period the mean body weight was 530 g. Food intake had been maintained at a constant level of 28 g/day (390 mg nitrogen) for about 4 months. For 30 days prior to the beginning of treatment the mean daily N excretion had been 364 mg. Postulating a fecal N of 8-9% of intake(14), the rats were in balance. The slow weight gain that continued was probably due to addition of fat with no appreciable protein deposition.

Comparison of the anabolic potencies of androstanolone and MAD with that of NTCP was undertaken because of recent interest in these compounds as anabolic steroids(3,5). Four rats were injected for 10 days with 2 mg androstanolone once daily, 4 with 2 mg MAD, and 4 with .25 mg NTCP. All 3 compounds caused decreases in N excretion lasting throughout the injection period (Fig. 3). Following withdrawal of treatment all animals returned to pre-injection N levels, and the androstanolone and MAD treated rats had transitory periods of N loss before leveling off at pre-injection levels. The relative magnitudes of the changes are seen in Fig. 3 and Table II.

NTCP was the most potent of the 3 compounds tested. The N retention effect of NTCP persisted for several days after withdrawal, while the rats receiving the other 2 compounds returned to or above pre-injection levels immediately following withdrawal. The total N retained by the NTCP-treated rats was about 1.7 times that retained by the androstanolone-treated animals and 2.7 times that retained by those receiving MAD.

Comparison of single injections of NTCP and nortestosterone. It has been shown(8) that NTCP has greater myotrophic activity than unesterified nortestosterone when the rat levator ani is used as an activity index. To confirm this difference by another test was desirable for characterization of NTCP and also for establishing parallelism between 2 animal tests.

An experiment was therefore performed in which N excretion was followed in 2 groups of animals treated with single injections of NTCP and nortestosterone. Two groups of 4 castrate male rats weighing a mean 424 g (372-542) at the beginning of the fore-period were treated with 2 mg of NTCP or nortestosterone in .1 ml cottonseed oil. A single injection of nortestosterone failed to produce a positive response. The single injection of NTCP produced a substantial depression of N excretion for about 10 days (Fig. 4).

Discussion. NTCP appears from these and previous(8) data to be one of the most potent anabolic steroids reported to date. Although it is difficult definitely to fix its N-retaining potency in comparison with TP because of the lack of differential in response to graded doses, one can speculate that it is roughly equivalent to TP. NTCP is more potent than androstanolone, a result consistent with Kochakian's findings(12,15) that androstanolone had less N-retaining potency than TP. NTCP is obviously a more potent N-retaining agent that MAD.

The fact that esterification with cyclopentylpropionic acid greatly improves the anabolic activity of nortestosterone is firmly established. The present data confirm the earlier levator ani data from this laboratory(8).

Summary. 1. In the adult castrate rat, oil injections of 19-nortestosterone cyclopentylpropionate produced decreases in urinary N excretion comparable with those obtained with testosterone propionate. 2. This compound caused greater retention of N than was observed with parenteral administration of me-

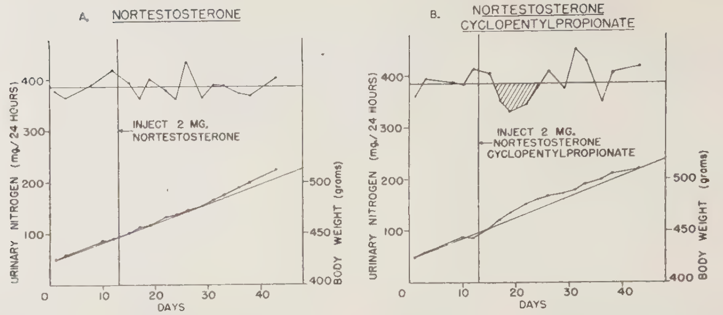

FIG. 4. Urinary total nitrogen and body weight changes of rats given single injections of (A) nortestosterone or (B) NTCP. Shaded area indicates nitrogen retention.

thylandrostenediol or androstanolone when given at ⅛ the dose of the 2 latter compounds. 3. 19-nortestosterone cyclopentylpropionate in a single injection of 2 mg produced retention of N lasting several days; unesterified nortestosterone, similarly given, did not result in significant N retention.

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Effect of Aluminum Hydroxide Gels on Experimental Hypercholesterolemia and Atheromatosis in Chicks.* (21087)

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It has been reported that aluminum hydroxide gel reduced cholesterolemia, delayed the onset of atheromatosis, and produced muscular weakness in chicks fed cholesterol(1,2). These effects were attributed to possible adsorption phenomena by alumina gel in the small intestine. The significance of these observations made it of interest to further investigate them.

Method. The regimen for producing atheromatosis was that of Dauber and Katz(3). One hundred White Leghorn cockerels certified to be free of salmonella infection were

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* Presented at meeting of Fed. Am. Soc. for Exp. Biol. and Med., Atlantic City, N. J. Apr. 12-16, 1954.

TABLE I. Experimental Design and Weight Changes (9 Groups).

Treatment group	No. of chicks	Treatment	Wt (g)	
			Initial	Final
A	12	CD*	434	1601
B	12	+ 5% CSO	454	1678
C	10	CSO + 0.5% Ch	405	1647
D	10	+ 3% AHG (Am)	478	1320
E	10	+ Ch + 3% AHG (Am)	438	1124
F	10	+ 3% AHG (B-Gel)	499	1621
G	10	+ Ch + 3% AHG (B-Gel)	463	1585
H	12	+ Ch + 0.2% boric acid	468	1643
J	10	+ 2 mg CA† IM/day	431	1483

* CD = Control diet; CSO = Cotton seed oil; Ch = Cholesterol; AHG = Aluminum hydroxide gel (Am = Amphojel®); CA = Cortisone acetate.

† Cortone® Merck.

divided into 9 groups according to the outline in Table I. The reasons for including some of the diets are obvious. Cortisone and boric acid were also included; the former because of the reported effects on blood cholesterol, and the latter because of its tendency to accumulate in fat depots(4) and its possible effects on fat metabolism(5,6). The control diet consisted of Purina chick starter mash to which was added 2.5% cod liver oil and 0.1% chick granite grit. The cholesterol was suspended in cottonseed oil[†] and then mixed into the control diet. B gel[‡] and Amphojel®,[‡] which is a mixture of B gel with another alumina gel, were incorporated into the basic diet and passed through a fine screen to remove lumps. The chicks were housed in wire cages and given food and water *ad libitum*. The daily food consumption and weekly weight changes were recorded. Total cholesterol levels were determined weekly on blood obtained from the alar vein by a modification of Bloor's method(7). To facilitate sampling each group was divided into 2 equal subgroups. The blood samples within each subgroup were pooled for cholesterol determinations. The values for the 2 subgroups were averaged. At the end of 15 weeks the chicks were decapitated and exsanguinated. Heart, lungs, liver, kidneys, spleen, pancreas, adrenals, thyroids, testes, and parts of the small intestine were removed. The organs were weighed, fixed in formalin, and prepared for histological examination. Relative organ weights were compared statistically by analysis of variance for unpaired data(8). The aorta was freed to the bifurcation and cut at the level of the last rib; the thoracic portion was severed at the heart and the abdominal portion at the level of the renal arteries. The sections were mounted on corks and the intima stained with oil Red O according to the method of Lilli(9). They were graded without prior knowledge of treatment. The gross grading was correlated with the microscopic grading. Frozen sections stained with oil Red O from representative levels were taken at random to prevent identification of labels and graded 1 to 4. Grades 1 and 2 represent small and moderate amounts respectively of fat-stained material without displacement of the endothelium; grade 3, plaques with irregular elevations of the endothelium; and grade 4, ulcerated plaques.

Results. Effect of treatment on hypercholesterolemia. All groups on the cholesterol diet developed hypercholesterolemia after 2 weeks of treatment. This persisted throughout the experiment except in the group receiving Amphojel® and cholesterol, (Table II) in which the levels returned to normal at the fourth week and remained in this range until the treatment was altered. The failure to maintain hypercholesterolemia was preceded by decreased food consumption which eventually resulted in inanition with the clinical signs of muscular weakness, diarrhea, and small combs. Similar symptoms were also seen in the group receiving only Amphojel®. In the ninth week the regimen for both groups was changed so that they received the basal

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[†] Puritan Brand—manufactured by Procter and Gamble, Inc.

[‡] Manufactured by Wyeth Laboratories, Inc.

TABLE II. Effect of Treatment on Total Blood Cholesterol Levels and Food Consumption (9 Groups).

	Week of experiment															
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
T.B.C.	67	68	67	69	64	60	53	61	82	82	75	59	81	64	77	77
F.C.	.5	.7	.9	.9	.9	.9	.9	.9	1.0	1.2	1.3	1.5	1.8	2.0	2.1	2.3
T.B.C.	69	68	76	58	60	61	48	60	92	88	75	63	80	73	87	73
F.C.	.5	.7	.9	.9	.9	.9	1.0	1.0	1.1	1.3	1.4	1.6	1.8	2.1	2.2	2.4
T.B.C.	69	77	131	111	98	126	95	119	173	172	138	105	114	110	120	96
F.C.	.6	.7	1.1	1.1	1.2	1.2	1.3	1.3	1.5	1.7	1.9	1.9	2.1	2.0	2.1	2.0
T.B.C.	86	54	70	56	54	49	39	46	55	88	89	96	85	74	88	98
F.C.	.7	.8	1.0	.9	.8	.7	.7	.5	.4	.8	.9	.9	1.2	1.3	1.4	1.4
T.B.C.	77	105	172	149	86	92	66	64	58	85	139	98	113	112	126	172
F.C.	.7	.8	1.0	1.0	.9	.8	.8	.5	.4	.5	.9	1.0	1.2	1.2	1.4	1.6
T.B.C.	85	58	70	53	59	52	42	47	67	92	114	83	84	70	98	98
F.C.	.7	.8	1.1	1.1	1.1	1.2	1.2	1.2	1.3	1.4	1.4	1.4	1.5	2.2	2.3	2.3
T.B.C.	74	66	214	119	129	161	135	112	194	201	189	125	116	117	138	126
F.C.	.5	.5	.9	.9	.8	1.0	.9	1.0	1.0	1.3	1.3	1.5	1.7	1.8	1.9	1.8
T.B.C.	80	134	196	151	153	160	124	141	194	201	189	125	116	117	138	126
F.C.	.5	.6	.9	.9	.9	.9	.9	.9	1.0	1.2	1.4	1.4	1.6	1.7	1.7	1.9
T.B.C.	69	93	137	124	114	117	92	118	199	172	124	104	121	119	138	136
F.C.	.6	.8	1.0	1.1	1.2	1.2	1.3	1.3	1.4	1.4	1.5	1.5	1.8	1.9	2.1	2.2

T.B.C. = Total blood cholesterol, mg %. F.C. = Food consumption, kg/bird/wk.

diet for 2 consecutive days per week, resulting in the disappearance of all symptoms of inanition and accompanied by persistent hypercholesterolemia in group E.

Changes in the cardiovascular system. With the exception of group H all cholesterol-fed chicks showed a significant decrease in relative heart weights (g/kg body weight). There was no significant difference in the heart weight of the boric acid-cholesterol and the control groups. The results of the microscopic grading of aortic lipid content are found in Table III. There was a high degree of correlation between the microscopic and gross grading. Lesions were seen in the control chicks; cholesterol feeding resulted in a marked increase in severity of lesions. Neither of the alumina gels, boric acid nor cortisone acetate lessen the atheromata induced by the cholesterol feeding.

Gross and histological changes exclusive of the cardiovascular system. A significant increase in weight and fat deposits were seen in the liver of all chicks receiving cholesterol. There was also some increased fat deposition in the spleen, kidneys, adrenals, and pancreas of the animals receiving cholesterol. Chicks receiving Amphojel® had retarded development of the testes which was associated with decreased comb weights. Sections of the combs from the boric acid-treated group stained by the Ritter-Oleson(10) method showed decreased mucopolysaccharides. Boric acid administration also resulted in the appearance of dark brown subcutaneous fat which appeared normal histologically.

Discussion. Our observations that alumina gel *per se* did not prevent hypercholesterolemia or atheromatosis in cholesterol-fed chicks do not agree with those of Rodbard, Bolene, Peck, and Katz(1), and Rodbard and Bolene-Williams(2). In chicks receiving only the cholesterol diet 50% of the lesions were grades 1 and 2; those receiving alumina gel and cholesterol 75% of the lesions were grade 3 and 4. Our data indicate that the lower cholesterol values in group E were the result of decreased food consumption rather than to an adsorption phenomenon in the small intestine. These findings agree with those of Horlick and Katz(11) who demonstrated that the

TABLE III. Degree of Atheromatosis in Aortas (%). 9 groups.

Thoracic					Abdominal				
0	+	++	+++	++++	0	+	++	+++	++++
55	46					64	27	9	
42	58				50	50			
50	38	13				13	38	25	25
	100					83	16		
		60	20	20				60	40
	44	56			67	33			
		17	42	42		17	25	50	8
	27	18	36	9		27	27	18	27
		44	33	22		22	22	44	11

blood level of cholesterol in chicks was proportional to the cholesterol intake.

The muscular weakness syndrome(2) is commonly seen in chicks suffering from malnutrition(12) and may be a manifestation of encephalomalacia produced by inadequate diets, particularly those with vitamin deficiency and 2% fish oil(15). Our data indicate that the normal cholesterol values and the symptoms of this syndrome seen in the Amphojel®-cholesterol treated chicks were due to decreased food consumption rather than to a systemic effect of aluminum; from the ninth week on when the food intake became adequate no symptoms were present although the aluminum ingestion was increased 2-fold. The groups receiving B gel did not reject the diet throughout the experiment and did not develop the paralysis throughout the prolonged feeding of large amounts of aluminum.

Our chicks did not develop hypercholesterolemia as response to stress (inanition) which differs from the response of rabbits and humans(7,13,14). The significance of the findings that boric acid prevented the decreased heart weight that results from the administration of cholesterol in chicks cannot be evaluated from the present work; neither can the apparent decrease of mucopolysaccharides in the comb nor the brown color of the subcutaneous fat. The latter may be a confirmation of earlier reports(5,6).

Summary. 1. As long as the diet was ingested the addition of 3% aluminum hydroxide gel did not prevent hypercholesterolemia or atheromatosis in cholesterol-fed chicks. Neither did such a diet result in inanition or paralysis. 2. Normal cholesterol values, inanition, and paralysis developed with rejection of the diet. The stress of inanition did not produce hypercholesterolemia in chicks. 3. Neither cortisone acetate nor boric acid influenced the development of arterial lesions, although the latter had some effect on lipid deposition.

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Studies on Inhibition of Tyrosinase. (21088)

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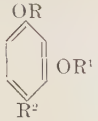
A number of substances including thioureas, cyanides, sodium azide, and hydroxyquinolines are known to poison phenoloxidase. Of these compounds, phenylthiourea(1,2) is probably the most potent inhibitor and, according to Chodat(1), prevents the *in vitro* activity of potato phenoloxidase at a concentration of 1 x 10^{-3}. Using a particularly sensitive method, Mayer, Grimm and Kull(3) were able to detect antiphenoloxidase activity of phenylthiourea at a concentration as low as 1 x 10^{-10} using tyrosine, β-(3,4-dihydroxyphenyl)-L-alanine (DOPA), epinephrine or norepinephrine as substrates. The antiphenoloxidase activity of thioureas and certain other substances is attributed to their ability to form complexes with copper, the essential metal component of the enzyme. The observation that these compounds exhibit similar inhibitory activity against other metal-containing enzyme systems supports this thesis. A second group of phenoloxidase poisons has been claimed to act by a different mechanism of enzyme inactivation, namely, competitive inhibition. To this group belong p-nitrophenol(4), 4-nitrocatechol(5), several 4-acyl tyrosines(6) and m-hydroxybenzoic acid(7). Except for the convincing kinetic demonstration of the competitive action of m-hydroxybenzoic acid presented by Warner(7), the mode of action of the other compounds within this group has not been supported by experimentation but inferred from the structural similarity between substrate and inhibitor. A large number of related aromatic compounds were studied which might on the basis of structural similarity, compete with the substrate for the enzyme.

Method. The filter paper method for the measurement of phenoloxidase activity described by Mayer, Grimm and Kull(3) was used. In this procedure the compounds to be tested for enzyme inhibition are dissolved in an appropriate solvent such as water, alcohol or chloroform, with the addition of hydrochloric acid or sodium hydroxide to dissolve bases or acids as required. Serial dilutions of these solutions are deposited in 0.05 ml portions upon large filter paper sheets. After drying, a phenoloxidase-substrate mixture is sprayed upon the paper in a stream of nitrogen. The enzyme-substrate mixture contains potato phenoloxidase mixed with equal parts of saturated solutions of L-tyrosine, 0.2% DOPA, epinephrine or norepinephrine in Sorensen buffer pH 7.0. The wet sheet is immediately suspended in a humid atmosphere and observed for color changes. Melanin formation rapidly proceeds through the various color changes, starting with the bright red hallachrome and terminating with a gray-black color when tyrosine or DOPA are used or red-brown in the case of epinephrine or norepinephrine. Interference with the oxidation is quantitatively indicated by round white spots. Since the extent of visible inhibition at the hallachrome stage is often considerably greater than the final degree observed when melanin formation has been completed, two endpoints may be established: that of a maximal but transient or temporary inhibition; and that of the definitive inhibition. Unlike the inhibitors, substances representing potential enzyme substrates produce spots darker in color than the developed melanin background. *Substances investigated.* Approximately 400 compounds including simple and substituted phenols, diphenols, aminophenols, amines and diamines were investigated. It is not possible to list all the substances tested and only those yielding the most interesting results are recorded in Table I.

Results. As seen in Table I, resorcinol at a concentration of 5 x 10^{-4} inhibits melanin formation from tyrosine or DOPA. This is in agreement with Baur's(8) findings. The monobenzoate and monostearate derivatives were 20 times more active than free resorcinol. Neutral resorcinol derivatives were inactive, whereas the diacetate had the same activity as resorcinol. The highest degree of

TABLE I. Inhibition of Potato Phenoloxidase (Tyrosinase) by Various Resorcinol Derivatives.

Chemical compound



R	R¹	R²	Name	Lowest conc. inhibiting enzyme
		(A) Unsubstituted resorcinols:		
H	H	H	—	5×10^{-4}
H	C_6H_5CO	H	-monobenzoate	1×10^{-6}
H	$C_{17}H_{35}CO$	H	-monostearate	1×10^{-6}
H	CH_3CO	H	-monoacetate	5×10^{-4}
H	C_2H_5	H	-monoethyl ether	—
H	CH_3	H	-monomethyl ether	—
CH_3CO-	CH_3CO-	H	-diacetate	5×10^{-4}
CH_3-	CH_3-	H	-dimethyl ether	—
C_2H_5-	C_2H_5-	H	-diethyl ether	—
C_6H_5CO	C_6H_5CO	H	-dibenzoate	—
		(B) Substituted resorcinols:		
H	H	Cl	4-Chlororesorcinol	1×10^{-8}
H	H	-CHO	2,4-Dihydroxy-benzaldehyde	5×10^{-4}
H	H	C_6H_{13}-	4-n-Hexylresorcinol	5×10^{-4}
H	H	3,5-di(2′,4-dihydroxyphenyl-triazinyl)	2,4,6-Tri-(2′,4-dihydroxy-phenyltriazine)	1×10^{-4}
H	H	CH_3CO-	Resacetphenone	5×10^{-4}
H	H	p-nitrophenylazo-	p-Nitrophenylazoresorcinol	5×10^{-4}
H	H	CH_2CHNH_2COOH	2,4-Dihydroxyphenylalanine	1×10^{-4}
H	H	COOH	2,4-Dihydroxy-benzoic acid	1×10^{-3}
H	H	m-nitrophenylazo	4-(3′-Nitrophenylazo)-resorcinol	—
H	CH_3	CH_2CHNH_2COOH	2-Methoxy-4-hydroxyphenyl alanine	—
CH_3	H	COOH	2-Hydroxy-4-methoxybenzoic acid	1×10^{-3}
		(C) Resorcinol-like compounds:		
			Orcinol	5×10^{-4}
			Phloroglucinol	1×10^{-3}
			Naphthoresorcinol	5×10^{-4}
			2-Nitroresorcinol	1×10^{-2}
			2-Aminoresorcinol	—
			3,5-Dihydroxyphenylalanine	—

inhibition was obtained with 4-chlororesorcinol. An activity in concentrations as low as 1 x 10^{-8} to 1 x 10^{-9} was recorded; thus indicating an activity of 2000 to 20,000 times greater than resorcinol. Other substitutions in the 4-position resulted in considerably lower activities or even complete abolition of enzyme blocking activity.

These results obtained with various simple 4-substituted resorcinols prompted the examination of isomers of the physiological substrate DOPA (a 4-substituted catechol), including d,L - 2,4 - dihydroxyphenylalanine.* During the preparation of the latter compound Lambooy(9) described the syntheses of various isomers of DOPA as well as the inhibition of tyrosinase by the 2-4-isomer. As seen from our table we obtained results similar to those of Lambooy. However, the 2,4 DOPA had only 1/10,000 of the activity of 4-chlororesorcinol.

Other simple derivatives of mono- or diphenols which displayed activities identical with that of resorcinol (5 x 10^{-4}) were orcinol, naphthoresorcinol and hydroquinone monobenzylether (a known melanin inhibitor).

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* These substances were prepared by Dr. R. Mizzoni, Chemical Research Laboratories, CIBA, Summit, N. J.

Phloroglucinol, 2-nitro- or 2-amino resorcinol and d,L-3,5-dihydroxyphenylalanine were inactive. Hydroquinone, m- and p-hydroxybenzoic acids yielded slight inhibition, while phenol, pyrogallol and m-methyl anisole had no activity. Catechol, a known substrate for the enzyme, produced dark gray spots of a melanin-like substance in concentrations as low as 1 x 10^{-5}.

The strongest inhibitor in a series of aromatic amines was m-aminophenol with an activity of 1 x 10^{-6}. p-Aminophenol was 20 times less active, whereas o-aminophenol was inactive. Of the 3 phenylenediamines only p-phenylenediamine proved to be a powerful enzyme inhibitor. Aniline, and several of its chlorinated derivatives, as well as toluidines showed little if any activity.

In order to compare quantitatively the activities of these new phenoloxidase poisons, a number of compounds previously known to be strong phenoloxidase inhibitors were examined. Mono substituted thioureas, such as the phenyl-, p-phenetyl-, and p-butoxy-phenylthioureas, showed activity ranges between 1 x 10^{-6} to 1 x 10^{-9}. Their efficacy was thus comparable to that of 4-chlororesorcinol. Definitely less potent was the most active representative of a group of 8-hydroxyquinolines. These compounds were effective in a dilution of 1 x 10^{-5}.

Discussion. Resorcinol derivatives probably substitute for the substrate or replace it to block the normal reaction. Support for this hypothesis is afforded 1) by the observation that the activity of 4-chlororesorcinol appears to be strongly dependent on the substrate concentration and 2) by the fact that 1,3-dihydroxybenzene derivatives are not likely to inactivate the enzyme by chelation with copper. Experiments are now in progress to analyze more specifically the possible competitive nature of the inhibitory mechanism of these compounds.

Summary. Using a previously described spot-spray technic, more than 400 simple, aromatic compounds were investigated as inhibitors of tyrosinase. 4-Chlororesorcinol inhibited melanin formation from tyrosine, DOPA or epinephrine at a concentration of 10^{-9}; it was thus 20,000 times more potent than resorcinol.

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Age and Susceptibility to Neurotropic Influenza Virus.* (21089)

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Numerous reports have appeared in the literature on the relation between the age of a given host and its susceptibility to various infectious agents. Mice, guinea pigs, and rabbits have been among the experimental animals most commonly used in these investigations. In addition, chick embryos in various stages of development, newly hatched chicks and adult chickens have frequently been employed. Many viral agents have been studied in this connection, among them the viruses of rabies and pseudorabies, eastern

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TABLE I. Incidence of Encephalitis in Mice Following Intraperitoneal Injection of NWS Virus.

Age (days)*	1	2	3	4	5	6	7	8	9	10	11	15	16	
Mortality	8/8	10/10	6/6	8/8	7/7	9/9	10/10	8/8	23/23	8/8	9/9	6/6	5/5	
% survivors	0	0	0	0	0	0	0	0	0	0	0	0	0	
Mean day of death†	3.9	5.5	5.2	6.0	5.1	5.3	4.9	5.3	4.0	4.1	5.0	5.5	6.0	
Age (days)*	17	18	19	20	21	22	23	24	25	26	27	28	29	35
Mortality	7/7	6/7	1/7	3/6	55/94	4/7	0/6	0/6	1/6	3/7	1/7	3/5	0/12	3/10
% survivors	0	14	86	50	42	43	100	100	83	57	86	40	100	70
Mean day of death†	6.1	5.5	6.0	7.7	5.9	7.0	—	—	6.0	9.3	11.0	9.0	—	11.0

* At time of inoculation. † Excluding survivors.

and western equine encephalomyelitis, influenza A, lymphocytic choriomeningitis, vesicular stomatitis, poliomyelitis, rabbit pox, and yellow fever. The great majority of published reports indicates that resistance to experimental viral infection increases with increasing age of the host; the degree of susceptibility of the very young animal or extent of resistance of older animals varies somewhat with the type of host, the particular tropism of the virus and the route of inoculation. A few exceptions to this apparent pattern of decreasing susceptibility with increasing age are to be found in the literature. For example, Sabin(1) demonstrated that more Lansing poliomyelitis virus was required to produce infection in newborn mice than in adult mice, that the incubation period was prolonged in suckling mice, and the survival time after onset of paralysis was shorter in weaned mice than in suckling mice. The excellent review by Sigel(2) summarizes reports in the literature up to 1952 on the subject of age and susceptibility to infection.

The present experiments were designed to determine the relation between age and susceptibility of mice to a neurotropic strain of influenza virus (NWS) inoculated peripherally. Stuart-Harris(3) has found that this virus is pathogenic for mice when inoculated subcutaneously up to the 10th day of life, but that after this time the virus is no longer effective except after intracerebral injection.

Materials and methods. Virus. The Stuart-Harris neurotropic variant of WS influenza virus (NWS) was used in all experiments. This strain evolved from the non-neurotropic WS virus after passage in chick embryo allantois, chick embryo brain, and, finally, by serial intracerebral passage in weanling mice(4). It has been maintained in this laboratory by serial passage in 10-day-old embryonated eggs. The allantoic fluids were harvested 48 hours after inoculation and, after determination of hemagglutinin titers, were stored at –10°C until used. Allantoic fluids from egg passage numbers 8, 9, 12, 15, and 18, having hemagglutinin titers of 1:192 to 1:384, were used in these experiments. Undiluted fluid was used for all mouse inoculations, the dose being 0.05 cc for mice 11 days of age and younger, 0.1 cc for mice 15 days of age and older. *Mice.* Pregnant Swiss mice (Webster strain) were obtained from commercial breeders. Each mouse was kept in a separate box and the date and time of parturition were recorded. In a few instances postpartum mice were obtained and in these cases the date of birth of each litter had been noted by the breeder. *Determinations of virus content.* Brains of infected mice were removed aseptically, emulsified without abrasive and made up as a 10% suspension with sterile broth-saline solution containing 100 units of penicillin and 5 mg of streptomycin per cc. Three mice were killed and their brains pooled for each determination. Serial 10-fold dilutions were made and 4 eggs inoculated by the allantoic route with 0.1 cc of each dilution. After 48 hours incubation at 36.5°C the allantoic fluids were harvested and the presence of virus determined by the capacity of individual fluids to agglutinate an equal volume of 1% washed chicken erythrocytes. The egg-infective titers of the pooled brain emulsions were calculated according to the Reed-Muench method for determining 50% endpoints(5).

Two types of experiments were carried out. In the first, mice from one to 11, 15 to 29, and 35 days of age were inoculated intraperitoneally with NWS virus. These mice were observed for a period of 15 days and the onset

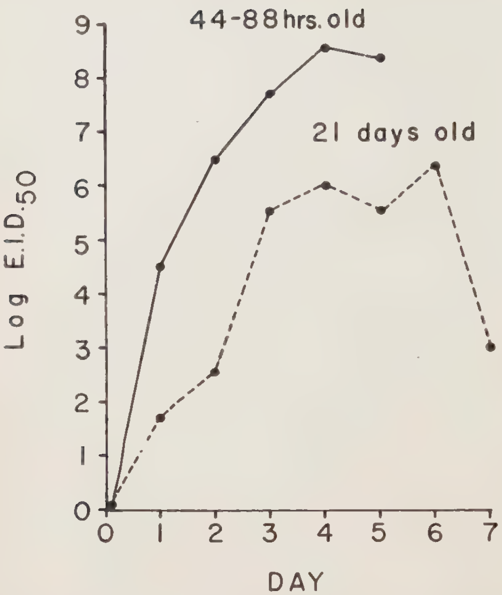

FIG. 1. Growth curves of NWS virus in infant and weanling mouse brain after intraperitoneal injection. Each point based on 3 animals; 0 day = 3½ hr after injection.

of paralysis and the day of death recorded. The other experiment was designed to compare the virus growth curves in the brains of infant and adult mice. Accordingly, 2 groups were inoculated with NWS virus (H.T. 1:256): in one group the mice were 44-88 hours old, and in the other 21 days old. Three mice from each group were killed 3½ hours after injection and an equal number at daily intervals thereafter. The experiment was continued for 7 days in the case of the 21-day-old animals; none of the infant mice survived beyond the 5th day.

Results. Table I shows the mean day of death at each age and the percentage of mice in each group that survived an intraperitoneal injection of virus. It can be seen that none of the 124 mice under 18 days of age at the time of inoculation survived the infection, whereas only 80 of the 180 mice between 18 and 35 days of age succumbed. Varying degrees of resistance were seen in the older age groups. There was no uniform increase in number of survivors with increasing age. Groups of animals between one and 17 days of age showed a mean day of death ranging between 3.9 and 6.1 days. In contrast to this, death occurred on an average of 5.5 to 11 days after injection of mice 18 days of age and older. All of the 24 mice in the 23-, 24-, and 29-day-old age groups survived.

A comparison of the daily virus titers in the brains of mice 44-88 hours and 21 days old is shown in Fig. 1. No virus was demonstrable in either group 3½ hours after injection (0 day). Subsequent to this, the virus titer of the brains of the younger age group rose more rapidly and to a higher level than that of the older animals. Each day the level of infectivity of infant mouse brain exceeded that of adult mouse brain by at least 10^2 EID_{50}; 48 hours after infection the difference was 10^4 EID_{50}.

Summary. In mice under 18 days of age, encephalitis and death invariably follow intraperitoneal injection of large doses of neurotropic-WS influenza virus. Older mice show varying degrees of resistance to infection. In general, decreased susceptibility is characterized by longer incubation periods and high proportion of survivors. The virus titer in infant mouse brain rises more rapidly and attains a higher level than in adult mouse brain. In addition, the degree of susceptibility may also be a function of the number of virus particles actually reaching the brain after intraperitoneal injection.

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Biocytinase, an Enzyme Concerned with Hydrolytic Cleavage of Biocytin. (21090)

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Biocytin refers to a conjugate of biotin isolated from yeast extract and identified as ε-N-biotinyl-L-lysine(1). Biocytin is available as a source of biotin for most biotin-requiring microorganisms, for example *Lactobacillus casei.* Biocytin is unavailable as a source of biotin for a few biotin-requiring microorganisms, for example, *Lactobacillus arabinosus.* Preliminary experiments have indicated that the ability of a microorganism to utilize biocytin as a source of biotin is correlated with the occurrence of an enzyme that cleaves biocytin yielding biotin as one moiety (2). The amount of enzyme present in such bacterial extracts as judged by the amount of biocytin hydrolyzed, was very small and rendered experimentation difficult. With the availability of synthetic biocytin in quantity (3), and with the indication that it is safe for administration in man(4), it has been administered to patients in an effort to discover some therapeutic use for this agent. In connection with these studies it has been found quite unexpectedly that blood and urine samples from such individuals appeared to contain biotin rather than biocytin as determined by differential microbiological assay. Subsequent study has shown that there exists in blood, and presumably other tissues as well, an enzyme in considerable quantity that is active in hydrolyzing biocytin to yield biotin.

Since the linkage involved in the conjugation of biotin with lysine (ε-acylation) appears to be unique as far as compounds derivable from natural material are concerned, it is suggested that a previously unrecognized enzyme may be involved in this hydrolysis. Biocytinase is suggested as a tentative name for this enzyme.

Methods. Enzymatic hydrolyses were carried out in open glass tubes without shaking at 37° for 1 hour in a constant temperature water bath. The basal reaction mixture usually consisted of 1 ml of 0.1 M phosphate buffer of pH 6.6 containing 0.01 γ of added biocytin as biotin per ml and 0.2 ml of a 1:10 dilution of blood, plasma, or serum in distilled water. Where variations in pH were studied this was accomplished by varying the proportions of M NaH_2PO_4 and M Na_3PO_4 used to prepare the buffer. In some preliminary experiments, especially where comparatively large amounts of blood were used, no buffer was employed beyond that of the blood itself. Where addenda were studied they were dissolved in the buffer-biocytin mixture if neutral or were added to the buffer-biocytin mixture in the form of neutralized solutions if acid or alkaline. Usually the blood samples studied were collected with sufficient heparin to prevent clotting. Citrated blood was also satisfactory. The enzymatic reactions were terminated by autoclaving the tubes for 5 minutes or by immersing in a boiling water bath for 10 minutes. The reaction mixtures were then diluted to 20 ml and aliquots assayed microbiologically for biotin with *Lactobacillus arabinosus*(5). Suitable controls established that the biocytin, phosphate buffer, blood, plasma, serum, and addenda studied were essentially free of interfering biotin. It was also demonstrated that the various addenda studied, in the concentrations employed, did

TABLE I. Data Demonstrating Quantitative Hydrolysis of Biocytin by Biocytinase of Plasma.

Enzymatic reaction time, min.	Hydrolysis of biocytin, %
0	4
2	46
5	84
10	98
15	100
30	107
45	101
60	99

Reaction mixtures: 1 ml biocytin sol. in water (0.01 γ biocytin as biotin/ml). 1 ml of normal human plasma. Enzymatic activity was stopped at indicated times by immersion of appropriate tube in boiling water bath for 10 min.

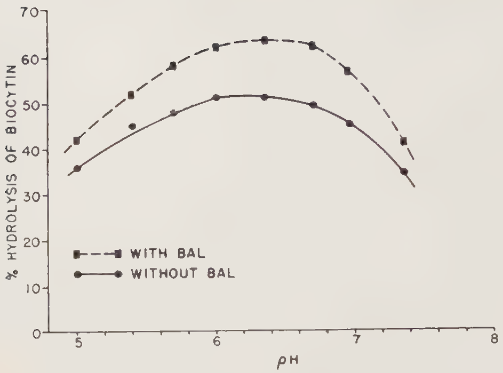

FIG. 1. pH optimum of biocytinase. Reaction mixture: 1 ml biocytin solution (0.01 γ biocytin as biotin/ml) in 0.1 M phosphate buffer of the indicated pH, 0.2 ml of a 1:10 dilution of whole blood in distilled water. BAL where used was present in the biocytin-buffer mixture to saturation. Reaction stopped by autoclaving tubes at 120°C for 5 min.

not influence the response of *Lactobacillus arabinosus* to biotin. Data are presented in terms of the per cent of the 0.01 γ of biocytin hydrolyzed under the conditions of a particular experiment.

Results. The experiment summarized in Table I demonstrates that biocytin is quantitatively hydrolyzed under appropriate conditions of time and enzyme concentration. For more critical studies of the reaction the enzyme concentration was lowered and the reaction time arbitrarily set at 60 min. Data summarized in Fig. 1 demonstrated that the pH optimum is in the range of 6.0-6.7. Additional experiments in which "tris" (tris-(hydroxymethyl) aminomethane) buffer was employed showed that no other maximum exists up to pH 9. Stability studies of biocytinase in which the enzyme content of blood samples stored at 5°C was determined showed that the enzyme appeared to vary in an unpredictable manner such as to suggest a "cyclic" type of activity. In an effort to "stabilize" the reaction, additions of the following compounds were tested and found inactive: niacinamide, manganese sulfate, yeast extract, glycine, magnesium sulfate, glucose, adenosine triphosphate, catechol disulfonate, 'Sequestrene', oxygen, nitrogen, carbon dioxide, ascorbic acid, cystine, potassium ferricyanide, potassium ferrocyanide, and sodium chlorate. Addition of any one of the following *sulfhydryl compounds* to the reaction resulted in consistent "high" values: thiomalate, thioglycollate, glutathione, and BAL (2,3-dimercapto-1-propanol). Some data demonstrating this effect are summarized in Table II. BAL because of its high activity, low solubility and neutrality was found most convenient to use. Stability studies in which the biocytinase activity of a sample of whole blood stored at 5° was determined at intervals with and without the addition of BAL to the reaction mixture are summarized in Fig. 2. It is apparent that essentially constant and high enzymatic activity of the blood was shown for a period up to 12 days. On the other hand, the activity determined without added BAL varied in an unpredictable manner as previously described.

Although a comprehensive study of the structural specificity of the substrate essential for biocytinase activity was not carried out, some data with biotinamide were obtained. In one experiment involving comparable quantities of the two substrates 47% biotinamide and 32% biocytin were hydrolyzed. In another experiment 56% biotinamide and 45% biocytin were hydrolyzed. If the two compounds are hydrolyzed by the same enzyme then biotinamide is acted upon at a slightly higher rate.

TABLE II. Effect of Certain Sulfhydryl Compounds on the Activity of Biocytinase.

Exp. No.	Addendum	Hydrolysis of biocytin
1	0	27
	Glutathione, .1 mg	32
	1.0	51
	5.0	59
2	0	25
	Thioglycollic acid, .01 mg	26
	.1	40
	.5	56
	BAL (saturated)	77
3	0	24
	Thiomalic acid, .1 mg	27
	1.0	38
	2.0	44
	BAL (saturated)	67

Reaction mixtures: 1 ml pH 6.63 0.1 M phosphate buffer containing 0.01 γ of added biocytin as biotin/ml. Addenda as indicated. 0.2 ml of a 1:10 dilution of whole blood in distilled water. Enzymatic activity stopped after 1 hr by autoclaving tubes at 120°C for 5 min.

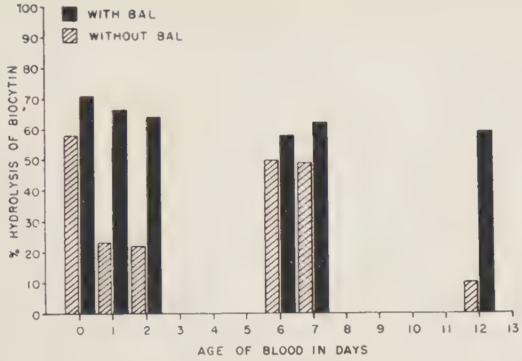

FIG. 2. Stability of biocytinase in stored blood. Reaction mixtures: 1 ml biocytin sol. (0.01 γ biocytin as biotin/ml) in 0.1 M pH 6.6 phosphate buffer, 0.2 ml of a 1:10 dilution of whole blood in distilled water. BAL where used was present in the biocytin-buffer mixture to saturation. Reaction stopped by autoclaving tubes at 120°C for 5 min.

The distribution of biocytinase between plasma and the cellular components of blood has not been extensively studied. In one experiment a sample of whole blood gave 66% hydrolysis of biocytin while the plasma derived from the same sample of blood gave 85% hydrolysis. It would appear that the biocytinase is largely present in the plasma although some activity must be present in the red or white cells to account for the distribution of activity observed.

Discussion. The enzymatic nature of the hydrolysis of biocytin by blood appears to have been established. The essential nature of an appropriate sulfhydryl compound for consistent maximal activity of the enzyme has been pointed out. The optimum pH of biocytinase is within the range of that common to most animal and microbial enzymes. The possible identity of biocytinase with a known amidase, peptidase, or acylase has not been established in this work. It would appear that some concentration and purification of the enzyme is essential before such a point could be established. It has previously been reported that biocytin is unaffected by commercial preparations of pepsin, trypsin, papain, takadiastase, polidase, or mylase(6).

Simple calculations show that there is enough biocytinase in the blood alone of an average adult to hydrolyze approximately 3 mg of biocytin in one hour. The body of an average normal human adult probably does not contain more than 1 mg of biotin in various "combined forms"(7). In addition, if all the biotin contained in a typical diet were in the form of biocytin, only about 0.25 mg calculated as biotin would be consumed per day(8). Thus it is apparent that there may be considerable "physiological pressure" for the conversion of biocytin to biotin. The significance, if any, of this phenomenon remains to be explored.

Summary. The existence in blood of an enzyme in relatively large amount, that accomplishes the hydrolysis of biocytin (ε-N-biotinyl-L-lysine) tentatively termed biocytinase has been pointed out. The enzyme has a pH optimum at 6.0-6.7 and requires for optimum effect the presence of an appropriate sulfhydryl compound.

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Steroids in Adrenal Venous Blood of the Dog: Venous-Arterial Differences Across the Adrenal.* (21091)

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In a previous communication from this laboratory the isolation of 14 fractions from dog adrenal venous blood was reported(1). A number of these substances may enter the gland by way of its arterial blood supply and cannot be considered secretory products. A comparison of the concentrations of the isolated substances in the affluent and effluent blood of the adrenal has yielded information on this important problem.

Methods. Mongrel male dogs were anesthetized with sodium pentobarbital. Adrenal venous blood was collected from the left lumboadrenal vein (the adrenal vein was ligated between the adrenal gland and the inferior vena cava). At the same time and at approximately the same rate of flow, arterial blood was collected from a femoral artery. Arterial blood pressure, recorded by means of a mercury manometer connected to a cannula in a carotid artery, was maintained at or near normal levels by the infusion of pooled dog blood at a rate approximately equal to the rate of bleeding. Heparin was injected intravenously to prevent clotting. The collected blood was diluted 1:1 with water and extracted twice with chloroform. The chloroform extracts were evaporated and the residues partitioned between hexane and 70% ethanol. The 70% ethanol fractions were chromatographed on paper by the method of Burton *et al.*(2); propylene glycol and toluene were the stationary and mobile phases, respectively. The design of chromatography was essentially that described in a previous communication from this laboratory (1). For quantitative analysis each chromatogram was divided at right angles to the length of the strip into 1- or ½-inch cuts, and the steroids were eluted from individual cuts with methanol. The methanol solutions were analyzed by 1) absorption in the ultraviolet, 2) reaction with phenylhydrazine, and 3) reaction with sulfuric acid. In 3 experiments, one liter of adrenal venous blood and one liter of arterial blood were collected over 4 to 6 hours. In 2 of these experiments, an additional liter of arterial blood was collected to which 17-hydroxycorticosterone and corticosterone were added to estimate recovery of added steroids and to test the possibility that degradation of the major steroid components occurs during chromatography. In each experiment, the adrenal venous blood, the arterial blood and the arterial blood containing added steroids were extracted and partitioned at the same time and chromatographed together in order to minimize the effects of minor variations in technic.

Results. 1) *Adrenal venous blood.* In an earlier study(1), 14 fractions were isolated by paper chromatography from the chloroform extract of a 3500-ml sample of dog adrenal venous blood. In Exp. I of this study (Table I), 13 fractions were isolated and shown to be similar to the corresponding fractions isolated in the previous study in regard to 1) relative position on the chromatograms, 2) type of reaction with phenylhydrazine, and 3) absorption characteristics in the ultraviolet. Fraction 14 was not detected. In Exp. II (Table I), 12 fractions were isolated; one of these fractions covered an area on the chromatogram usually occupied by Fractions 3 and 4; another covered an area usually occupied by Fractions 13 and 14. In Exp. III (Table I), 14 fractions, corresponding to those described in a previous report, were isolated.

The results of these 3 experiments indicate that, in general, the steroid patterns of adrenal venous effluent from different dogs are qualitatively similar. However, certain differences are to be noted. For example, in Exp. I, the adrenal did not produce Fraction 11 (11-de-

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TABLE I. Substances Found in Arterial and Adrenal Venous Blood of the Dog.

		(1) Conc. in adrenal venous blood, $\mu g/100$ ml			(2) Conc. in arterial blood, $\mu g/100$ ml			(1)−(2) Adrenal contribution, $\mu g/100$ ml		
		Exp.								
Fraction No.*	Identification	I	II	III	I	II	III	I	II	III
1	Not identified	3.6	4.8	2.8	4.2	2.3	2	−.6	2.5	.8
2	*Idem*	†	†	†	†	†	†	†	†	†
3	"	6.8		4.1	5.6		2	1.2		2.1
			9.5			2.4			7.1	
4	"	4.4		1.9	3.6		1.4	.8		.5
5	"	†	†	†	†	†	†	†	†	†
6	17-hydroxycorticosterone	66.4	259	294.4	5.4	3.8	12.2	61	255.2	282.2
7	Not identified	10.9	14.5	18.1	10.3	15.6	15.9	.6	−1.1	2.2
8	*Idem*	12.5	4.9	10.4	.7	0	0	11.2	4.9	10.4
9	"	†	†	†	†	†	†	†	†	†
10	"	†	†	†	†	†	†	†	†	†
11	11-desoxy-17-hydroxycorticosterone	1.6	39	46.1	1.9	3.3	1	−.3	35.7	45.1
12	Corticosterone	40.5	116	120	3.5	6.4	7.2	37	109.6	112.8
13	Not identified	15		23.4	0		11.6	15		11.8
			44.5			28.8			15.7	
14	*Idem*	0		29.2	0		10.3	0		18.9

* Fraction number refers to those fractions described in an earlier publication(1), and are arranged in order of polarity.

† These substances could not be quantitated in terms of μg steroid. They appeared to be present in the same concentrations in arterial and adrenal venous blood, as estimated by U.V. absorption in the instances of fractions 2, 5, and 10 and by sulfuric acid chromogen in the instance of fraction 9.

soxycorticosterone), whereas in Exp. II and III this substance was secreted in significant amounts. Furthermore, it is not yet possible to state the exact number of steroids present in adrenal venous blood. A number of the fractions isolated may be a mixture of steroids. Indeed, recent work on Fraction 8(3) indicates that this fraction consists of at least 2 steroids. Final statements as to similarities and differences in the steroid patterns of various dogs should be deferred until such time as it is possible to more completely characterize the individual components of the mixture.

2) *Comparison of adrenal venous blood and arterial blood.* (*Table I*). Comparison of the concentrations of the steroids found in femoral arterial blood with those found in adrenal venous blood provides information on the secretory activity of the adrenal cortex. *Fraction 6* (17-hydroxycorticosterone) is clearly of adrenal origin, since the concentration of this steroid in adrenal venous blood was many times that in arterial blood in each experiment. *Fraction 7* (an unidentified steroid which absorbs at 230 mμ, exhibits a 340 mμ maximum with phenylhydrazine and shows maxima at 240, 285, and 370 mμ in sulfuric acid) was the largest single steroid component in arterial blood in each experiment. There was no evidence that the blood concentration of this substance was increased by passage through the adrenal. In view of the relatively high concentration of this steroid in peripheral blood further study is warranted. It is noteworthy that neither the method of Nelson *et al.*(4) nor that of Sweat (5) for the analysis of steroids in peripheral blood would detect the presence of this substance. *Fraction 8,* although present in low concentrations, was clearly of adrenal origin in each experiment. The major constituent of Fraction 8 is a steroid which absorbs at 240 mμ and gives a 400 mμ maximum with phenylhydrazine reagent. This fraction has been found to be 25 times as potent as desoxycorticosterone in producing sodium retention in the adrenalectomized rat(6). The sodium-retaining activity of this fraction does not appear to be associated with the major com-

TABLE II. Comparison of Steroid Content of Arterial Blood to Which 17-Hydroxycorticosterone (Exp. II, 100 μg/100 ml; Exp. III, 150 μg/100 ml) and Corticosterone (50 μg/100 ml) Had Been Added with That of Arterial Blood.

Fraction No.*	Identification	(1) Conc. in arterial blood, μg/100 ml		(2) Conc. in arterial blood with added steroids, μg/100 ml		(2)–(1) Recovery, μg/100 ml	
		Exp.					
		II	III	II	III	II	III
1	Not identified	2.3	2	4.9	2.4	2.6	.4
2	*Idem*	†	†	†	†	†	†
3	"	2.4	2	4.7	1.7	2.3	–.3
4	"		1.4		1.6		.2
5	"	†	†	†	†	†	†
6	17-hydroxycorticosterone	3.8	12.2	100.4	140.3	96.6	127.9
7	Not identified	15.6	15.9	23	10.4	7.4	–5.4
8	*Idem*	0	0	0	0	0	0
9	"	†	†	†	†	†	†
10	"	†	†	†	†	†	†
11	11-desoxy-17-hydroxycorticosterone	3.3	1	2.4	2.6	–.9	1.6
12	Corticosterone	6.4	7.2	33.6	48.1	27.2	40.9
13	Not identified	28.8	11.6	21.8	8.1	–7	–3.5
14	*Idem*		10.3		11.9		1.6

* Fraction number refers to those fractions described in an earlier publication(1), and are arranged in order of polarity.

† These substances could not be quantitated in terms of μg steroid. They appeared to be present in the same concentrations in arterial and adrenal venous blood, as estimated by U.V. absorption in the instances of fractions 2, 5, and 10 and by sulfuric acid chromogen in the instance of fraction 9.

ponent, but rather with a substance which exhibits an absorption maximum at 240 mμ but which does not react with phenylhydrazine to yield a derivative which absorbs at 400 mμ (3). This finding is an example of a steroid of great metabolic importance present in very low concentration in adrenal venous blood.

Fraction 11 (11-desoxy-17-hydroxycorticosterone) was not present in higher concentration in the adrenal venous blood in Exp. I, although it was clearly so in Exp. II and III. The role of this biologically inactive steroid in the adrenal secretion is not clear. The variability in its production by the glands remains unexplained. *Fraction 12* (corticosterone) was present in the adrenal venous blood in a concentration many times that in arterial blood in each experiment. This steroid is clearly of adrenal origin. *Fraction 13* (an unidentified steroid which absorbs at 230 to 240 mμ, exhibits a 340 mμ maximum with phenylhydrazine reagent and exhibits maxima at 280 and 370 mμ in sulfuric acid) was present in higher concentrations in adrenal venous blood than in arterial blood in Exp. I and III. In Exp. II, it was not separated from Fraction 14, although the concentration of Fraction 13 plus 14 was higher in adrenal venous blood than in arterial blood in this experiment. *Fraction 14* was not present in the adrenal venous blood in Exp. I, and was not well resolved in Exp. II; in Exp. III it appeared to be present in higher concentrations in adrenal venous blood. This fraction is a non-polar substance or mixture of substances which chromatographs close to the front in the solvent system, absorbs at 230 to 235 mμ and gives a 290 mμ peak in sulfuric acid. Further study is required to elucidate the nature of this material and the role of the adrenal in its production.

Fractions 1, 3, and 4 are substances which are more polar than 17-hydroxycorticosterone, which react with phenylhydrazine to give maxima in the region of 400 mμ and which fluoresce in sulfuric acid; they were present in both adrenal venous and arterial blood in low concentrations. Their status as adrenal secretory products cannot be definitely established at this time. The small positive arterial-venous differences in concentration of Fractions 1 and 3 plus 4 in Exp. II suggests that they were secreted at a low rate. However, when arterial blood to which 17-hydroxycorticosterone (Exp. II, 1000 μg; Exp. III, 1500 μg) and corticosterone (500 μg in each experiment) had been added was analyzed and the concentrations of the various fractions compared to those of the control samples of arterial blood (Table II) a small positive difference in concentrations of Fractions 1 and 3 plus 4 of Exp. II was found. The possibility that these fractions may be formed by the degradation of one or more of the major fractions during processing cannot be ruled out.

3) *Recovery of 17-hydroxycorticosterone and corticosterone added to arterial blood.* In Table II are presented the results of the recovery studies. 17-Hydroxycorticosterone added to blood (100 μg/100 ml in Exp. I and 150 μg/100 ml in Exp. II) was recovered in 96% and 86% yields, respectively. Fifty μg/100 ml of corticosterone was added to blood in both experiments and was recovered in yields of 60% and 84%, respectively. Other than the very slight absolute increase in the concentrations of Fraction 1 and Fraction 3 plus 4 in Exp. II (see above) there is no evidence of change in the pattern of steroids. These experiments indicate that none of the major fractions which have been described in adrenal venous blood is an artifact derived by degradation of the major constituents during extraction and chromatography.

Summary. Concentrations of steroids in arterial and venous blood of the adrenal have been determined. Those steroids whose concentrations in the blood are increased by passage through the gland are considered to be adrenal secretory products. Three steroids are consistently of adrenal origin: 17-hydroxycorticosterone, Fraction 8, and corticosterone. In 2 of 3 experiments 11-desoxy-17-hydroxycorticosterone was contributed to the blood by the adrenal gland. A steroid less polar than corticosterone (Fraction 13) also appeared to be an adrenal secretory product in 2 of 3 experiments. Five substances (Fractions 2, 5, 7, 9, and 10) are clearly not secreted by the adrenal gland, since they were found in the same concentrations in arterial blood as in adrenal venous blood. The status of 4 substances (Fractions 1, 3, 4, and 14) as adrenal secretory products has not been established. 17-Hydroxycorticosterone and corticosterone, when added to arterial blood, are recovered without substantial alteration in the pattern of steroids present in such blood.

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Effects of Neonatal Anoxia on Maze Learning in Rats. (21092)

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It is generally agreed that severe oxygen deprivation at the time of birth can result in a variety of behavior disorders involving intellectual deficit. These effects may be due solely and directly to neonatal anoxia(1,4,6,8). An alternate possibility is that failure to breathe normally at birth is a secondary effect resulting from congenital neurological defects which are also responsible for the psychological abnormality often associated with early oxygen deprivation.

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In an attempt to study the behavioral effects of neonatal anoxia in otherwise normal individuals a few experiments on animals have been conducted(3,5). Investigations of the learning ability of rats rendered partially anoxic shortly after birth have revealed some impairment, but statistically significant decrements have not been produced. In none of the earlier studies was oxygen deprivation complete, and in all instances the newborn animals had been breathing normally for some period of time before partial anoxia was induced. The present investigation was conducted to examine the long-term effects of anoxia induced before respiration had begun, to compare the effects of varying periods of total anoxia, and to measure these effects in a highly sensitive test.

Method. Induction of anoxia. Female rats were mated in pairs to fertile males so that both members of a pair would come to term at approximately the same time. At the expected time of parturition the animals were kept under constant observation and as soon as one female delivered her litter, the second animal was sacrificed by a sharp blow on the head and the abdomen was then opened to expose the uterus. Fetuses were left in the uterus for the desired period of anoxia after which they were removed and the membranes cleared away from the head so that respiration could begin. The anoxic period was taken to be the time elapsing between the cessation of maternal respiration and the initial inspiration shown by the experimental animal. As soon as respiration was established the young were placed with the female whose litter had just been born, and her own offspring were removed. This method of providing a recently parturient foster mother was necessary to insure a high rate of successful adoptions. Thirty-three experimental and 23 control animals were used. *Learning tests.* The Hebb-Williams closed field maze was used to measure learning(2). This method of testing learning ability has been standardized and refined by Rabinovitch and Rosvold who found it more reliable than other mazes and more sensitive in reflecting deficits produced by small lesions in the cerebral cortex(7). The maze consists of a plywood box 3 feet square with the entrance alley and food compartment at opposite corners. Movable barriers are arranged in various patterns to create 6 different practice problems and 12 different test problems. Areas lying outside of the most direct path from the starting alley to the food compartment are considered error zones, and an animal's score consists of the total number of entrances into error zones in the course of 8 runs to the food on each problem. One unique and valuable feature of this device lies in the use of a succession of different problems which permits generalization or transfer from one pattern to the next. It might be said to measure the rat's ability to "learn to learn."

Testing schedule. When the subjects were 58 days old adaptation to the maze was begun. Animals were deprived of food for 24 hours and were subsequently fed only in the food compartment of the maze. The duration of the feeding period was adjusted for each individual so as to maintain body weight approximately 15 g below the weight at satiation. Different practice problems were presented twice each day until adaptation was complete. The criterion for adaptation was 9 runs to the food tray in not over 60 seconds on 2 consecutive problems. This criterion had been met by the time the rats were 65 days old, and at this point the 12 learning tests were begun. Rats were tested on two different problems each day for 6 days. Daily sessions were spaced 10 hours apart. Every animal was allowed 8 runs through the maze on each of the 12 problems, and for each problem the total number of errors made in reaching the food compartment was recorded. The testing procedure was held as constant as possible and the order in which the animals were tested was varied for each problem in an attempt to control the possibility that rats run late in a series might follow odor trails left by animals run previously.

Results. General effects of anoxia. It was found that a large proportion of the rats could survive to adulthood after a 20-minute period of anoxia at birth, but none of the animals prevented from breathing for 21 minutes or longer could be revived even temporarily. Within the lethal limit there was no clear cut relation between mortality and the duration of anoxia. Fifty-nine percent of the control animals survived to the termination of the investigation at 71 days. Within the experimental group, survival to 71 days after 1-10 minutes of anoxia was 69%, after 11-15 minutes it was 86%, and after 16-20 minutes, 72%. The great majority of deaths occurred within the first 48 hours or so after birth. No relation was found between the duration of anoxia and the weight and general health of the animals throughout the course of the study. The 33 experimental animals were subdivided on the basis of the duration of anoxia as follows: 1-10 minutes, 16 animals; 11-15 minutes, 7 animals; 16-20 minutes, 10.

Maze performance. Control animals made an average total of 178.4 errors on the 12 tests combined, and the comparable score for the experimental group was 194.0. This difference was significant at the .06 level of confidence (t-test, one tail). Within the experimental group there was no consistent relationship between the duration of the anoxic period and the number of errors made in the maze. *Control* rats made an average of 94.2 errors during the first 6 tests and 84.2 errors in the last 6. This difference was significant at the .05 level. In contrast, the average number of errors for experimental animals was 97.3 in the first 6, and 96.7 in the last 6 tests. This difference was not statistically significant. Average error scores for control and experimental rats did not differ significantly during the first 6 tests, but control animals were significantly better than experimental rats in the last 6 tests ($p = .04$). Although the experimental group as a whole was inferior to the controls, there was no statistically significant relation between duration of the anoxic period and progressive improvement during the 12 tests. Absence of such a relationship may have been due to the small number of cases in some of the experimental groups.

Summary and conclusions. Periods of anoxia ranging from 1 to 20 minutes were induced in unborn rats by preventing the initiation of respiration before removing the full term fetuses from the uterus. Beginning at 65 days of age, the experimental and control animals were tested on the Hebb-Williams closed maze. In a series of 12 standardized tests the experimental rats made more errors than the controls ($p = .06$). The inferiority of anoxic animals was most marked in the final set of 6 tests during which they were significantly inferior to the control group ($p = .04$). Control rats displayed progressive improvement in performance in successive maze problems, but no such improvement was evident in the records of experimental cases. It appears that oxygen deprivation at the time of birth tends to reduce the rat's ability to transfer prior learning to new situations. Within the experimental group, however, there was no relationship between the duration of the anoxic period and the extent of deterioration in maze performance with respect to either total scores or improvement in successive tests.

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Bacteriophagy of *Streptomyces griseus* as Revealed by Phase Microscope.*
(21093)

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Hofer(1) reported observation, but not photography of the lytic action of a rhizobium phage, as seen through the phase microscope. Since certain differences are apparent between his observations and our own with a different phage: host system, this note is offered. The host used is *Streptomyces griseus,* strain 4, and the phage is W2a isolated from soil here.

Methods. A suspension of spores was prepared by pipetting 3-4 ml of liquid medium (3 g yeast extract, 2.5 g glucose, and one g peptone per liter of distilled water) onto a 4-day slant culture on agar medium of similar composition. Approximately one ml of the suspension was removed to a small test tube and held 3 hours to allow incipient germination. A loopful of the germinating spore suspension was then added to about 0.2 ml of phage filtrate of approximately 5×10^7 particles per ml. When lysis was anticipated, wet mount preparations under No. 0 coverslips were set up under bright phase-contrast for observation over the critical period; the latent period with this phage : host system is about 1.5 hours. An American Optical (Spencer) phase microscope with turret condenser and Periplan 12X ocular was used. Light was provided by a Bausch and Lomb lamp with 6-volt ribbon filament regulated by a transformer. For the photomicrographs, a green (B) filter and Plus-X film were used. The camera was a Leica III-C with a Micro Ibso attachment.

Results. The general appearance of the cells at the stage used may be seen from the figures, showing the spore outlines from which protrude one or more short tubules or young hyphae. The beginning of lysis is indicated by less opacity and distinctness of outline, particularly at the tip and point of attachment of the hypha to the old spore body. It is to be noted too that the cells in the course of lysis do not appear larger in size than normal cells; they become merely hazy, granular and finally fade from sight. Following lysis of the young hyphal cell the old spore body persists for a time. Fig. 1, location *a,* shows a cell in early stage of lysis and just above it, at location *b,* one in late stage of lysis with the spore body still rather distinct. In succeeding Fig. 2-6 the cell at *a* is seen to be undergoing rapid lysis, since the time lapse between pictures is 5 minutes. Thus the time for disappearance of cell *a* is about 15 minutes from the normal appearance in Fig. 1 to complete lysis before Fig. 4. Traces of the spore body persist, however, nearly twice as long; Fig. 6 was taken 25 minutes after Fig. 1. Meanwhile another cell at *c* is undergoing lysis in Fig. 3-6.

The above phenomena of lysis are remarkable in their rapid sequence, realized in the study of living preparations. Contrary to the report of Hofer, we were unable to see phage particles attacking the cell surface with the optical system here used. Further there was no evidence of either swelling or bursting of the cell during phage multiplication. Although there is a granular stage of the cell (note Fig. 3, *a*), there is no residue suggestive of phage particles when lysis is complete.

The slow final lysis of the spore body suggests that it is of different composition from the young hyphal cell; perhaps it is a spore membrane or wall, slowly autolyzed rather than lysed by phage.

Summary. Lysis by phage of germinating spores of *Streptomyces griseus* has been observed and photographed under the phase microscope, bright phase-contrast. The time

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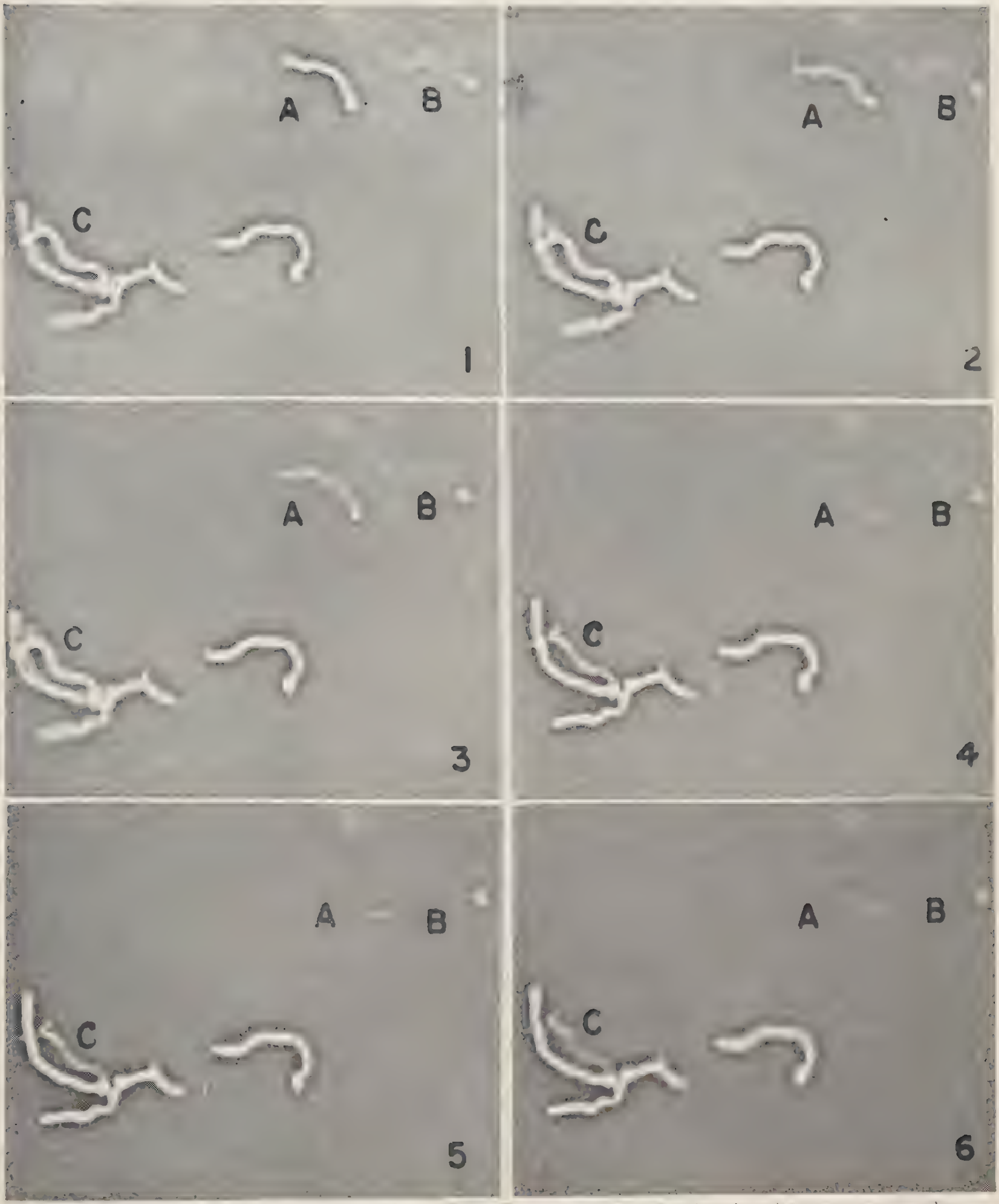

FIG. 1-6. Lysis of *S. griseus* by phage W2a. Photographs under bright phase-contrast, taken at 5 min. intervals. ×862.

required for complete lysis of a young hyphal cell is about 15 minutes.

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Liver as the Site of Synthesis of Nicotinic Acid from Tryptophane in Rats.

(21094)

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The dietary tryptophane is converted into nicotinic acid in the body of the rat(1). While some workers claim that this transformation takes place in the intestine by the activity of the intestinal micro-organisms(2,3) others have shown that it takes place in the tissues of the animal(4,5). On incubation of liver homogenates with tryptophane small amounts of nicotinic acid and quinolinic acid have been obtained, which indicate that possibly liver is the organ concerned in this transformation(6-9). Henderson and Ramasarma (10), however, did not confirm the above observation. In order to determine whether liver is the organ concerned in the synthesis of nicotinic acid from tryptophane in rats urinary excretions of some of the metabolites of nicotinic acid after administration of tryptophane were estimated in rats after their livers were poisoned by injection of carbon tetrachloride. The effect of incubation of livers from normal and carbon tetrachloride-treated rats with tryptophane on the production of nicotinic acid and quinolinic acid was also studied.

Experimental. Twenty-four hour urinary excretion of nicotinic acid, quinolinic acid, and N-methyl nicotinamide in normal and carbon tetrachloride injected rats. Rats, weighing 125-175 g, were housed in individual metabolism cages and urine collected in flasks containing one cc concentrated hydrochloric acid. They were fed a nicotinic acid-deficient diet (11) with which dl-tryptophane was mixed at the level of 1%. The daily urinary excretions of nicotinic acid, quinolinic acid, and N-methyl nicotinamide (N-MN) were estimated for one week. After this period all the animals were given a daily intraperitoneal injection of carbon tetrachloride (0.03 cc/kg), and the same metabolites were estimated in the urine. All the animals were weighed on alternate days and their food consumptions were noted. The results are given in Table I.

TABLE I. 24 Hr Urinary Excretion of Nicotinic Acid, Quinolinic Acid, and N-MN by 12 Rats before and after Injection of Carbon Tetrachloride. Avg of 6 days' excretion.

Carbon tetrachloride	Nicotinic acid (γ)	Quinolinic acid (γ)	N-MN (γ)
Before inj.	50 ± .09*	474 ± 67	603 ± 94
After 7 days inj.	29 ± .07	117 ± 29	187 ± 18

* Stand. error.

Incubation of liver homogenates with tryptophane. Two groups of rats (60-150 g in weight) were used. The first group received 1% dl-tryptophane-supplemented nicotinic acid-deficient diet only, while the second group received the same diet with a daily intraperitoneal injection of carbon tetrachloride for 7 days (0.03 cc/kg). Injection of carbon tetrachloride was given to the animals of the second group 2 days before they were placed on experimental diet. The animals were then killed and their livers quickly removed and homogenized in a chilled mortar with ice cold Krebs Ringer phosphate buffer solution of pH 7.4. Aliquots of the liver homogenates (400-500 mg) were taken in 2 weighed flasks. One of the flasks contained 2 cc Krebs Ringer phosphate buffer and 5 cc distilled water, while the second flask contained in addition 10 mg of dl-tryptophane in 5 cc solution. The flasks were quickly weighed after the addition of liver homogenate. The first flask was immediately boiled for half an hour and the second flask was incubated for half an hour at 37°C. After incubation the flask was boiled for half an hour; 2 cc of 50% trichloroacetic acid was then added to the flask and the volume made up to 25 cc and filtered. Ten cc filtrate was then taken for the estimation of nicotinic acid according to the method previously described (12). Another 10 cc portion was autoclaved for one hour with N sulfuric acid according to the method of Sarett(13), nicotinic acid was then estimated by the chemical method

TABLE II. Incubation of Liver Homogenate from Normal and Carbon Tetrachloride Treated Rats with Tryptophane. (Incubation period 30 min.)

	Nicotinic acid (mg %)		Quinolinic acid (mg %)	
	Without substrate	With substrate	Without substrate	With substrate
Normal (7)	14.33 ± 2.1	14.58 ± 1.8	50.94 ± 13.8	86.28 ± 12.8
Injected (7)	12.22 ± .9	12.10 ± 1.1	35.10 ± 10.7	52.90 ± 7.2
t	.07	.03	2.26	1.2

Figures in parentheses indicate the number of animals.

(12), and quinolinic acid values have been calculated by multiplying the increase of nicotinic acid by 5.8 as described by Sarett(13). The results are shown in Table II.

Discussion. It will be seen from Table I that the urinary excretion of nicotinic acid, quinolinic acid and N-MN was greatly decreased in rats injected with carbon tetrachloride, which damaged the liver. Incubation studies showed that livers of both groups of animals could not convert tryptophane to nicotinic acid. This observation agrees with that of Henderson and Ramasarma(10). It was observed that homogenized liver from normal rats could convert tryptophane to quinolinic acid more efficiently than the livers of animals receiving injection of carbon tetrachloride. This finding supports the observation of Wang *et al.*(7), but was in disagreement with works of Henderson(10). These studies indicate that liver plays a role in the transformation of tryptophane to nicotinic acid. It may be either the site for the synthesis of nicotinic acid or it might supply the essentials necessary for the conversion of tryptophane to nicotinic acid.

Summary. 1. Intraperitoneal injections of carbon tetrachloride in rats fed a dl-tryptophane supplemented nicotinic acid-deficient diet lowered urinary excretion of nicotinic acid, quinolinic acid and N-MN. 2. Liver homogenates of normal rats converted tryptophane to quinolinic acid more efficiently than that of carbon tetrachloride-treated rats. 3. It is suggested that liver is the principal site of synthesis of nicotinic acid and quinolinic acid from tryptophane.

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Development of Electrical Activity in Cerebral Hemispheres of the Chick Embryo.* (21095)

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Several authors have studied the EEG during both pre- and post-natal growth in rabbits (1-4), cats(5), guinea pigs(6,7) albino rats (8) and human foetus(9). The chick embryo is excellent material for recording and analyzing development of the electroencephalogram (EEG) as well as for comparison with morphologic, histochemical and other data obtained from study of cerebral hemispheres. The fairly simple structure of its nervous system, the uniformity in development, the possibility of determining exact age and facilities for using this animal for research have greatly helped in conducting this work.

Material and method. Seventy-three Rhode Island Red chick embryos varying in age from 9 to 20 days of incubation were used. Egg shell and membranes were pierced over the air chamber and the head made to protrude through the opening. Until the 14th day proper fixation of head is enough to obtain the EEG. Thereafter the embryo was inmobilized with curare (d-tubo-curarine chloride, 0.3-0.6 mg) in order to avoid recording artifacts due to muscular activity. The dorsal aspect of one or both hemispheres was uncovered by removing the parietal cartilage, the dura mater, and 2 to 6 electrodes consisting of Ag-AgCl-cotton wool-Ringer's solution were placed thereon. In some of our experiments an Ag-AgCl wire was placed in the beak as a reference electrode. Bipolar leads from the cerebral electrodes and/or "unipolar" leads with the common reference electrode were used. The *cerebral electrodes* cover the dorsal part of the hemispheres.† This wide zone includes, towards the rostral region and dorsomedially, the nucleus diffusus dorsalis and dorsolateralis, and dorsolaterally the thin lateral corticoid area L2. Caudally it includes the cortical areas, field d and field a + b + c. Two silver electrodes were introduced into the egg on either side of the body in order to record the electrocardiogram (EKG) and the artifacts due to movements of the embryo. A 2 beam cathode oscillograph with 4 stages for direct coupled amplification, a Grass 8 channel electroencephalograph and Offner 4 channel electroencephalograph were used. In the younger embryos high amplification ratios were used (up to 1 mm per microvolt). Embryos were placed at an oven at 38°C because room temperature exposure reduces EEG amplitude and frequency. Environment was generally kept saturated with moisture, as any drying of the brain considerably alters electrical activity, while electrode drying causes artifact. Under these conditions perfect vitality of the embryo can be kept for more than 24 hours as the EKG and EEG show. Strychnine activity was induced with strychnine sulfate crystals or with 1 mm square filter paper soaked in a saturated solution of this salt. In every case the strychnine was placed upon the dorsal aspect of the hemispheres near one of the electrodes. Metrazol was used with the same technic.

The following *controls* were made: a) electrodes fidelity was checked by joining them with a filter paper soaked in Ringer's solution and/or by making a record using the same amplification and position after the embryo death; b) movements causing serious artifacts were checked by means of the electrodes within the egg; and c) the vitality of the embryo by the EKG.

Results. 1. *"Spontaneous" electrical activity.* As the study of 64 embryos shows, "spontaneous" electrical activity appears on the 13th day of incubation. This activity is irregular with a mean frequency of 2.5 to 3 per sec., of low voltage (5-10 μv), sporadic, in short trains of waves, alternating with

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* This research was carried out in co-operation with the Departments of Histology and Embryology, of Physiology, Facultad de Medicina, and with the Institute of Endocrinology of Ministerio de Salud Pública, Montevideo, Uruguay.

† Topographical references are according to Kuhlenbeck(10).

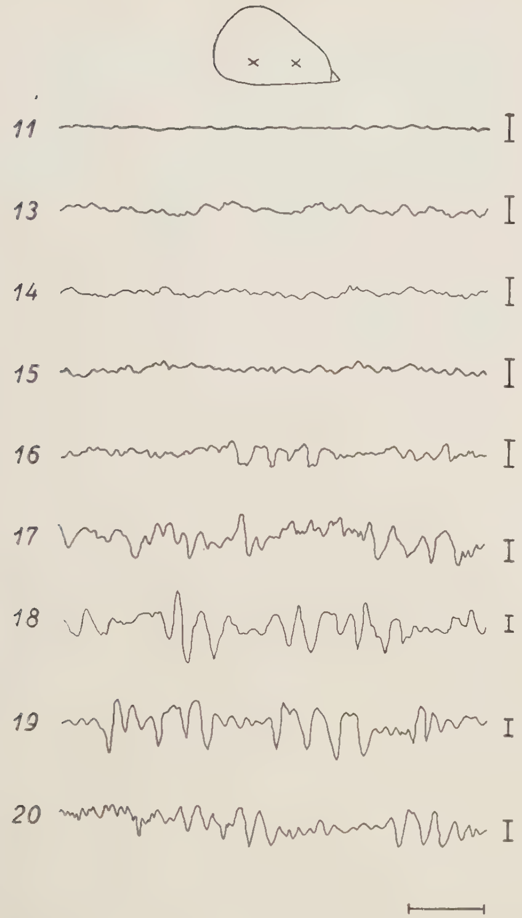

FIG. 1. *Beginning and development of the EEG in the chick embryo.* 11-20 days of incubation. Standardization: 50 microvolts. Time: one sec. In the upper part is shown the distribution of the electrodes upon the surface of the cerebral hemisphere; bipolar leads. 11: the small oscillations seen are produced by the apparatus. 13-15: ''early'' rhythm. 16: start of ''late'' rhythm. 17-20: further development of the ''late'' rhythm. For details, see text.

"silent" periods lasting several seconds. In no case was electrical activity observed before the 13th day (Fig. 1—11 and 13). EEG thus appears in relatively early stage of embryonic development the same as in guinea pigs(6,7), but unlike rabbits(2) and albino rats(8) where it appears later. On the 14th and 15th days there is little increase in frequency and voltage. The "silent" periods become continually shorter until, on the 15th day, they practically disappear (Fig. 1—14, 15). Low voltage and silent periods are also characteristic of EEG in early development phases in other animals(2,6-8) and in human foetus (11).

On the 16th day of incubation the pattern of the EEG changes considerably. High voltage wave trains, ranging from 20 to 100 μv, appear upon the preceding fundamental activity, showing a frequency of 3-4 per second. On the 16th day they are brief and sporadic. On the next 3 days (17th to 19th) they become progressively longer and more constant (Fig. 1—16-19). The appearance of other rhythm on the 16th day of incubation agrees with what is seen in the guinea pig and the rabbit in which at the end of fetal development more than one rhythm appears(2,6,7). In order to distinguish these 2 types of waves, we have given the name "early" rhythm to the low voltage waves appearing on the 13th day, and that of "late" rhythm to the slower high voltage waves observed from the 16th day. The main difference between these rhythms is the voltage. After the 16th day the "early" rhythm gradually increases in voltage and frequency until the 19th day. Meanwhile there is an increase in the number and duration of the "late" rhythm wave trains. The periods in which "early" rhythm is still visible becomes shorter. Voltage and frequency of "late" rhythm shows no significant changes. On the 20th day the EEG becomes dysrhythmic and the voltage decreases (Fig. 1—20). At this time there is considerable pulmonary respiration, and anoxia provoking embryo death in 1-2 hours is caused when curare stops respiratory movement.

At all ages electrical activity varies from place to place. The rhythms are not synchronous in the 2 hemispheres and voltages are different. In the dorsal aspect of the hemisphere the waves are more constant and of higher voltage rostrally.

Exposure of the brain of the embryo to a dry atmosphere for 15 to 30 minutes alters the EEG considerably inducing an epileptoid activity similar to that observed in adult mammals through drying of the brain(11). Random high voltage spikes appear at first, becoming more frequent with time. Subsequently regular discharges of spikes are seen, reaching a maximum and decreasing gradu-

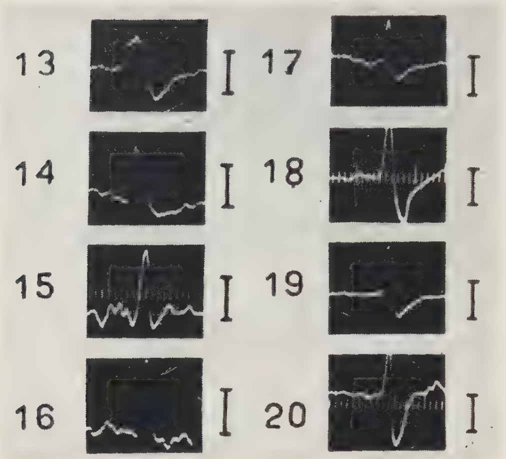

FIG. 2. *Development of strychnine activity in the chick embryo.* Incubation of 13-20 days. Standardization: 20 microvolts for 13-16 days, 50 for 17 days and 200 for 18-20 days. Time indicated in 15, 18 and 20, 50 millisec. (the same in all records). For explanations see text.

ally, while random spikes reappear. These discharges are repeated at intervals. This epileptoid activity may last as long as 24 hours without appreciable change. The 14th day is the earliest time at which this phenomenon has been observed.

Ischemia of the brain leads to the disappearance of electrical activity in 30 to 60 seconds, in some cases preceded by a transient increase in frequency and voltage, very marked at times and leading to a discharge of spikes.

2. *Electrical activity induced by strychnine.* In 45 embryos a study of the EEG changes due to local application of strychnine was made. The strychnine spikes appear on the 13th day, *i.e.,* at the same age that the "spontaneous" electrical activity(7). The same occurs in foetus of guinea pigs and in the newly born albino rat(8). The spikes are generally biphasic with a negative surface wave followed by a positive surface wave (Fig. 2). The negative wave is shorter than the positive, and is characteristic of these spikes, since it is always present and has approximately the same duration throughout a given experiment. In some cases the spikes are negative and single phased, polyphasic in others. The shape of the spikes is the same throughout a given experiment. The spikes are nearly always isolated, appearing at intervals, but occasionally may appear grouped together forming trains in which the frequency increases to a maximum and then decreases, constituting a spike discharge similar to that obtained by drying or ischemia. The spikes spread rapidly to the whole of the dorsal aspect of the homolateral hemisphere and to the opposite hemisphere. When the strychnine spike activity reaches a certain intensity, violent movements of the whole embryo ensue.

The strychnine activity varies considerably with embryo development. The duration of the spikes decreases as the embryo grows (Fig. 2) as in the case of the rabbit(3) and albino rat(8) in post-natal growth. The duration—age ratio curve of the daily averages is approximately exponential (Fig. 3). The duration of the negative wave is measured at the moment it crosses the isoelectric line, and the mean value is determined for at least 10 spikes. The extreme values found were 278 milliseconds on the 13th day and 51 milliseconds on the 20th. The amplitude of the spikes increases with age, although not so uniformly as the decrease in duration. The older the embryo, the greater the frequency of the spikes. On the 13th day there is usually an interval of over 5 minutes between spikes; on the 20th day there are 20-25 spikes per minute. This increase with age is not regular. The younger the embryo the greater the time that elapses between the local appli-

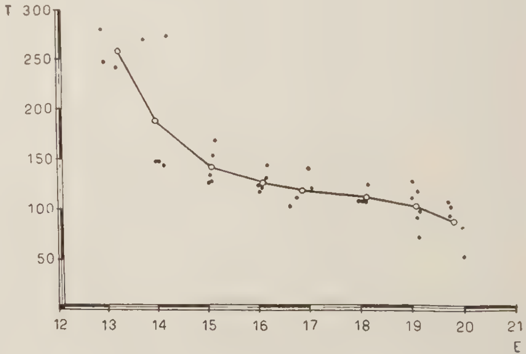

FIG. 3. *Strychnine activity in the chick embryo: duration-age ratio curve.* T: duration of negative phase of spike in millisec. E: days of incubation. Black dots: duration of the strychnine spikes in each experiment. White dots: general average duration on each day of incubation. For explanation see text.

cation of the strychnine and the appearance of the first spike, ranging from 50 minutes on the 13th day to 5 minutes on the 19th and 20th days.

The background electrical activity increased as a result of local application of strychnine in most of the experiments. Metrazol applied locally has the same qualitative effect as strychnine sulfate, but its action is weaker and not as constant.

Discussion. The "spontaneous" activity probably originates in the cell groups of the surface of the cerebral hemisphere. Bipolar leads from electrodes only 1-2 mm apart obviate the possibility of picking up the activity of deep structures. Besides, this "spontaneous" activity appears at the same time as the strychnine spikes, the origin of which has been shown in mammals to be in the superficial layers of the cortex(12). This fact is important, inasmuch as the rate of development of the deep nuclei is faster than that of the surface areas. The "spontaneous" electrical activity and that produced by drying are greatest in the dorso-rostral region. The diffuse dorsal and dorso-lateral nuclei are well developed at this level, consisting of 2 compact unstratified masses of cells, the forerunners of the neocortex of mammals(10). On the other hand, in the dorso-caudal region wherein the cortical areas a + b + c and d are already well developed, the electrical activity is not as great.

On the 12th day of incubation the nuclear differentiation of the telencephalon of the chick embryo can be regarded as completed. Later on, with the development of fibers, the centers acquire their ultimate structure and synaptic connections are established(10). It is precisely at this stage that the EEG appears and that the cells are enabled to discharge synchronously with strychnine stimulation. As the cells and their intercellular connections develop, "spontaneous" electrical activity becomes more evident and the capacity to synchronize increases. These facts agree with what has been found in other animals(3,7,8).

Various authors have established certain characteristics of the biochemical differentiations of the brain in the developing chick. The adenylpyrophosphatase (apyrase) activity of the brain in the chick embryo rises steadily from the 12th day of incubation on (13). The *ribonucleic acid* increases suddenly after the 13th day of incubation in the chick(14). Synthesis of phosphatides is especially marked from the 13th day of incubation on. The galactolipids increase suddenly between the 10th and the 13th day and the sphingomyelin appears between the 13th and the 16th day(15).

All these facts, ascertained by various investigators, have established that the electrical activity of the brain appears in the chick embryo at a critical time in the morphological and biochemical development of the cells. In opposition Nachmansohn(16) found that colinsterase increases suddenly just before hatching when, according to our experiments, electrical activity is already well developed.

Kuo(17) has analyzed various aspects of the physiological development of the embryo. From the 10th to the 14th day of incubation a considerable reduction in the massive movements of myogenic origin is observed and localized wriggling and jerking appear. This change in the patterns of the movements is very interesting as the EEG appears at this same stage, indicating a basic function of the central nervous system. From the 15th to the 18th day, rudimentary motor responses appear in answer to various sensorial stimuli (light, sound, rotation, and vibration). At this stage the EEG develops greatly and the "late" rhythm appears.

Summary and conclusions. 1. A study of the beginning and development of the EEG in 73 chick embryos is made. 2. "Spontaneous" electrical activity and that produced by strychnine appear simultaneously on the 13th day. 3. "Spontaneous" activity undergoes changes during the course of embryonic development. The "early" rhythm appearing on the 13th day gradually increases in frequency and voltage and becomes more constant. On the 16th day the high voltage "late" rhythm appears, becoming more constant on subsequent days. 4. Strychnine causes the appearance of spikes, which are generally biphasic, and increases the amplitude, frequency and constancy of the "spontaneous" activity in most experiments. 5.

With development of the embryo, the strychnine spikes become of shorter duration and higher voltage, pointing to a better synchronization of the neurones. Frequency increases and spikes begin to appear earlier as the embryo grows. 6. From the 14th day spike discharges are to be seen in the embryonic brain, similar to the epileptic discharges produced in man and animals by certain agents like anoxia, strychnine, metrazol, and drying. 7. The bioelectric development is compared with the histological, cytological, and biochemical development of the brain, and with the physiological activity of the embryo.

The author wishes to express his recognition to Professor Washington Buño, for suggestions and assistance.

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Interaction of Heparin with Auto-Agglutinins in Idiopathic Acquired Hemolytic Anemia.* (21096)

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Heparin was long considered the physiological regulator of the mechanism of blood coagulation(1,2). Later findings demonstrated its influence on plasma components belonging to the immune system, namely complement(3,4), and iso-agglutinins(5,6). It was also found that the antibody titer of rabbits sensitized against bacterial antigens increased manyfold upon previous or simultaneous administration of heparin(6). Astrup presented evidence which seemed to disprove the theory of heparin as a physiological anticoagulant. He showed that in no instance where "the tendency of blood to coagulate was greatly increased," could a rise in the blood heparin level be observed. This fact, with the other biological relations of heparin, has led him to advance the hypothesis that the "primary function of heparin in the organism should be looked for in connection with immunological processes"(7,8). Owren observed that the *in vivo* destruction of red blood cells in a patient suffering from acquired hemolytic anemia was retarded by large doses of heparin(9). Because of an increased bleeding tendency, the experiment had to be stopped after a few days, and this withholding of heparin resulted in a severe rise in the rate of cell destruction. No further comment as to the reason for using heparin and its possible mechanism of action was given, appar-

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TABLE I. Serological Findings in 3 Patients with Idiopathic Acquired Hemolytic Anemia.

	Titer of Coombs test		Agglutinin titers*								
			3°C		22°C		37°C				
Patient	Direct	Indirect	Alb.	Saline	Alb.	Saline	Alb.	Saline	Trypsin cells	Kolmer	Prothr. times†
R. R. (♀) Age: 17 A, CDEe/MN	1:128	1:2	1:1	1:1	1:8	Neg	1:32	Neg	1:1	Pos	17.2″
N. Y. (♀) Age: 35 O, cDEe/N	1:32	Neg	1:1	1:2	1:4	”	1:16	”	Neg	AC	14.5″
A. L. (♀) Age: 38 A, CDE . . .	1:256	”	Neg	1:4	1:64	1:2	1:64	”	1:1	Pos	16.2″

* The serial dilutions of serum were made using 0.1 patients serum plus 0.1 (20%) albumin or saline as a starting mixture. Same vol cell suspension was added to dilutions, incubated for 30 min. at temperatures listed, and subsequently centrifuged at 1000 rpm for 2 min.

† The normal prothrombin controls (100%) were 12.4″ and 12.5″.

ently because the experiment was "not wholly convincing . . ."

To our knowledge, the literature does not contain reports dealing with the interaction of heparin with auto-agglutinins. We would like, therefore, to present *in vitro* experiments, on 3 consecutive cases of idiopathic acquired hemolytic anemia, which appear to substantiate the possibility of such an interaction and to reemphasize the importance of heparin in immunologic processes.

Materials and methods. Commercial anti-human globulin serum (Ortho), bovine albumin (Armour), and Liquemin® (Heparin-Roche)† were used throughout. The concentrations of heparin employed ranged between 0.02-2.0 Toronto Units per 0.1 ml red blood cell suspension (5 x 10^5 cells). The characterization of the antibodies was performed by routine immunologic procedures. The cells were washed before performing the Coombs test, except where otherwise indicated. The cells of patient No. 3 were centrifuged only for the initial studies (600 rpm/one min.). In subsequent tests, the incubated mixtures were allowed to stand at room temperature for times ranging from 45 minutes to 6 hours. A "reverse" typing was necessarily performed on this patient, because of the almost immediate agglutination of her cells in any protein media. Heparin tolerance tests on plasma and whole blood were performed by the method of Waugh-Ruddick, as modified by Silverman (10). The diagnosis of iodiopathic acquired hemolytic anemia was made on the basis of severe anemia, reticulocytosis, elevated indirect serum bilirubin, spherocytosis, a positive direct Coombs test and the presence of auto-agglutinins in patients sera. Splenomegaly was present in all 3 cases; a careful survey revealed no apparent cause for the hemolytic process.

The studies here reported were made prior to any treatment or medication, except for one blood transfusion in patient No. 1. Patients Nos. 2 and 3 were relapse cases. Table I summarizes the serological findings of these patients on admission.

Results. When red blood cells from patient No. 1 and No. 2 were incubated with heparin for 10 minutes at room temperature and subsequently tested with anti-human globulin serum, it was found that they no longer agglutinated, thus giving a negative direct Coombs test. Coombs test titers performed with: a) heparin still present in the cell suspension, and b) removed by washing the incubated cells with fresh saline, gave the same results. Different concentrations of heparin

TABLE II. Effect of Heparin on Coombs Test Titer. Three patients.

		Cells incubated with heparin and subsequently washed				
Initial direct Coombs titer	Cells not washed after incubation with heparin	2.0 U	1.0 U	.5 U	.1 U	.02 U
1:128	Neg	Neg	Neg	Neg	1:1	1:8
1:32	”	”	”	1:1	1:4	1:32
1:256	”	See text . . .				

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† "Organon" inc. Orange, N. J.

TABLE III. Effect of Heparin on Albumin Agglutination Titer.

Dilutions:		1:1	1:2	1:4	1:8	1:16	1:32	1:64
Serum diluted with albumin alone (control)	R. R.	4+	4+	3+	2+	2+	+	Neg
	N. Y.	4+	3+	2+	2+	+	Neg	”
As above, cells washed with heparin prior to incubation	R. R.	—	—	—	Neg	—	—	—
	N. Y.				”			
Serum diluted with heparinized albumin. (Heparin conc. constant)*	R. R.	—	—	—	Neg	—	—	—
	N. Y.				”			
Heparinized serum diluted with albumin. (Decreased heparin conc.)	R. R.	Neg	Neg	+	2+	2+	+	Neg
	N. Y.	”		2+	2+	+	Neg	”

* 10 units/ml.

affected the Coombs titer differently, and a rough parallelism between heparin concentration and decrease in titer could be observed, as shown in Table II.

In the case of patient No. 3, in order to avoid centrifugation, the cells were not washed after incubation with heparin. 0.1 ml antihuman globulin serum dilution was added to 0.2 ml incubation mixture (*i.e.*, 0.1 cells plus 0.1 heparin or saline) and read at room temperature. While visible agglutination could be detected (dil. 1:4) after only 2 minutes in the control, reaching the 1:64 dilution at 8 minutes, the tubes containing 0.5 unit of heparin required 15 minutes to show visible agglutination, and the reaction stopped after 30 minutes at 1:4. In the tubes with 1.0 unit of heparin, only a questionable agglutination developed after 45 minutes. Negative results were also obtained when washed cells from patients No. 1 and No. 2 were resuspended in albumin dilutions of their own serum, to which a constant amount of heparin was added, or when the cells were "washed" *with heparin* prior to incubation with serum and bovine albumin dilutions. However, in serial dilutions made with a heparinized serum (*i.e.*, decreasing heparin concentration in respect to the constant number of cells present), a delayed agglutination became visible (Table III).

Because of the difficulties encountered in handling the cells of patient No. 3, a different procedure was used in her case. From a test tube containing the patient's clotted blood, the clot was carefully removed and immersed in another tube containing the patient's clear serum. The resultant cell suspension was divided into 2 parts. Saline was added to one (0.2 ml) and the same volume of heparin (containing 10.0 U/cc serum) to the other. The samples were then left at room temperature for 6 hours. A heavy agglutination occurred in the tube containing the cell-serum suspension with saline after less than 15 minutes, whereas the heparinized suspension did not show visible agglutination at the end of the 6-hour period. Since certain observations to be discussed below seemed to indicate a direct interaction between heparin and the auto-agglutinins, it became of interest to determine whether heparin activity was impaired simultaneously with the loss of agglutination. It was observed that a heparin solution previously incubated with above patient's red blood cells lost much of its anticoagulant activity against normal plasma. For this reason heparin tolerance tests were performed on the plasma and whole blood of patients No. 1 and 2. The results, showing abnormally high heparin tolerances despite the elevated prothrombin times (Table I) are illustrated in Fig. 1. A normal control was performed only on whole blood.

Discussion. It becomes apparent that while the agglutinin titer decreases with increasing heparin concentrations, no correlation with the initial strength of the Coombs reaction can be established. The red blood cells from patient No. 2 require more heparin in order to become desensitized to the same extent as the cells of patient No. 1 (Table II), although she has a lower Coombs titer. This phenomenon is reemphasized in the heparin tolerance curves, where again the patient with the lower titer has the higher heparin tolerance (Fig. 1). Thus, there seems to be a qualitative difference between the agglutinins

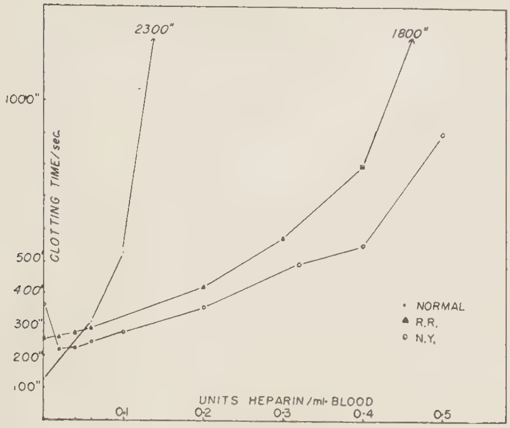

FIG. 1. Heparin tolerance test of two patients suffering from idiopathic acquired hemolytic anemia.

of these 2 patients, manifested by a difference in reactivity to heparin, and it is interesting to note that clinically the condition of patient No. 2 was more severe than that of No. 1. It is generally agreed that the Coombs reaction is no index of the gravity of a hemolytic episode. Whether the inhibition by heparin will prove to have any prognostic value awaits further accumulation of data. It was also shown that for the *in vitro* suppression of agglutination of coated red cells by antiglobulin serum or the patient's own serum, the presence of heparin is not necessary, provided the cells were previously incubated with it. From this follows that heparin does not inactivate or inhibit the antiglobulin serum or some "factor" in patient's serum, but reacts with the agglutinin itself. When one separates the cells previously incubated and washes them, the inhibition persists. At the same time, the cell-free heparin supernatant becomes almost inactive as an anticoagulant against normal plasma. Thus, the increased heparin tolerance of the blood could be due to the functioning of the agglutinin as a heparin inhibitor, at the expense of its own activity. Since heparin is closely related to hyaluronic acid (which was reported to yield upon depolimerization a distinctly anti-viral split product: glucuronolactone(11)), and possesses antithrombic and antihyaluronidase activity, similar to substances inhibiting competitively Newcastle disease and influenza virus hemagglutination(12), it could be included in the family of hemagglutinating-virus antagonists. The possibility of competition between auto-agglutinins and heparin should not be overlooked. However, the virus antagonists seem to inhibit agglutination *per se,* irrespective of the nature of the hemagglutinating factor, as evidenced by their action even on ABO and Rh antibodies(13). Heparin, on the contrary, does not seem to have any *in vitro* effect on isoagglutinins. Coombs tests performed on cells coated with iso-antibodies, (erythroblastotic cells and normal cells previously suspended in the serum of a patient having a 1:1024 anti-Rh titer), showed that treatment with heparin was ineffective in preventing agglutination‡(14). Therefore, whatever the mechanism of inhibition may prove to be, it seems specific for *auto*-agglutinins (or for a type of auto-agglutinins), and could become of diagnostic importance.

Concomitant with the inhibition of agglutination, a second reaction takes place, as suggested by the abnormal heparin tolerance curves (Fig. 1). Experiments to be reported showed that the prolonged prothrombin times of all 3 patients (Table I) were not due to a deficiency but to an inhibition. Using hyaluronidase(15) instead of heparin, a competition with the agglutinins for *proconvertin* was demonstrated. Such a competition, if valid also for heparin, may be the logical explanation for the initial correction of the recalcification time (patient N.Y.).

Finally, we would like to recall the relationship between proconvertin and complement, and the customarily encountered positive or anticomplementary Kolmer reactions (16) in acquired hemolytic anemia. The question arises whether this apparent "complexity" is but the manifestation of one and the same phenomenon, observed from different points of view; and that there need not be a contradiction between the old concept of Howell and the new concept expressed by Astrup in regard to the physiological role of heparin if one assumes that it acts on components common to both the immune system and the mechanism of blood coagulation.

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‡ Valid also for cells sensitized *in vitro* with anti D (albumin) typing serum.

Summary. 1. Heparin was found to inhibit *in vitro* the agglutination of red blood cells in 3 consecutive cases of idiopathic acquired hemolytic anemia. 2. Following incubation of patients cells with heparin, the auto-agglutination disappeared. The Coombs reaction also became negative while the anticoagulant activity of heparin greatly diminished. The heparin tolerance, tested in the first 2 cases, was abnormally high. 3. No interaction *in vitro* was observed between heparin and isoagglutinins. 4. The possible mechanism, specificity and clinical value of the reaction is discussed, and certain implications with respect to the clotting mechanism are pointed out.

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Use of Anthrone Reagent to Estimate Inulin in the Presence of Glucose.* (21097)

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Young and Raisz(1) have demonstrated that Dreywood's anthrone reagent can be used for the quantitative determination of inulin. They concluded that the anthrone method had many advantages over other procedures currently employed. Studies in our laboratory during the past 2 years have led to a similar conclusion although difficulty was found in applying the anthrone procedure for the estimation of inulin in the presence of glucose. The resolution of this problem, important for biological experimentation involving inulin, is the main consideration of the present study.

Direct anthrone technic for inulin. Morris (2) demonstrated that an intense heat needed to effect maximal color reaction was generated by the rapid addition of the anthrone reagent *directly* into an aqueous solution of most carbohydrates. With inulin, however, we found that the color formed with the anthrone reagent was maximal when we *suppressed* the heat of solution created by the direct intoduction of the anthrone reagent. This finding was applied to estimate microquantities of inulin in the following manner: Two ml quantities of standard aqueous solutions of inulin were introduced into Klett tubes which were immersed in a cool tap water bath for 10 minutes. Into the tubes 4 ml of 0.2% anthrone reagent were delivered rapidly by

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* This study was supported by grants from the Ciba Products Corp. and the Sadie Baron and George P. Wakefield Foundation.

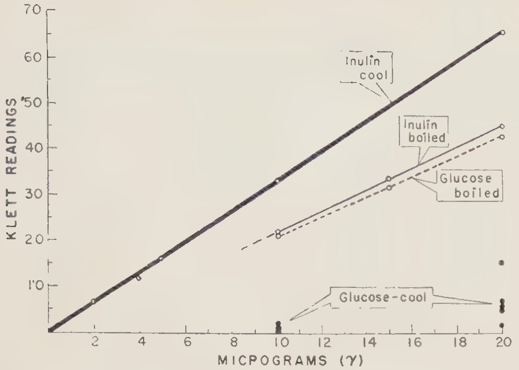

FIG. 1. Color formation by inulin and glucose with the "Direct Technic." Curve and dots labelled "cool" give Klett-Summerson readings ($\lambda = 620$ μ) when 4 ml of anthrone is blown rapidly into Klett tubes containing 2 ml aqueous solutions of inulin and glucose and cooled in tap water bath. Curves labelled "boiled" give Klett-Summerson readings after tubes were in boiling water bath for 10 min.

blowing the reagent out of 4 ml pipettes; the contents were thoroughly stirred with glass rods. After 2 or 3 minutes, the tubes were removed from the water bath, allowed to reach room temperature, and read in a Klett photoelectric colorimeter with a 620 mμ filter. The chief care required in the procedure was to obtain a consistent rate of delivery and thorough mixing of the anthrone reagent.

In Fig. 1 are shown *results* (labelled "cool") obtained with microconcentrations of inulin and glucose by this direct technic. Quantities of inulin from 2 to 20 μg led to color formation that was linear with the concentration. For a given concentration in this range, the optical density never varied more than 2% from one experiment to another. With glucose, however, the results were found to be totally inconsistent and unsatisfactory with this technic (Fig. 1, glucose-cool); the resultant optical densities varied with imperceptible and uncontrollable changes in the delivery of the anthrone reagent. Boiling for 10 minutes in a water bath resulted in a straight line curve for several concentrations of glucose, but it also lowered the sensitivity of the anthrone reaction for inulin as compared with the direct technic. This decreased sensitivity ruled out the boiling procedure except for large quantities.

Effects of alkali on inulin. The extreme simplicity and sensitivity of the direct technic for the estimation of small quantities of inulin made it worthwhile to explore methods to eliminate any accompanying glucose. Such a step was necessitated by the inconstant reactivity of glucose thus precluding the use of a "chromogenic blank" for correction even when a constant amount of glucose was present. Following the suggestion of Little(3), sodium hydroxide was employed to destroy glucose in the presence of inulin. This was attempted by adding 0.5 ml of 5 N NaOH to one ml quantities of aqueous standard solutions of inulin and glucose in Klett tubes. The tubes were heated in a boiling water bath for exactly 5 minutes, cooled, and neutralized with 0.5 ml of 5 N H_2SO_4. The anthrone reagent (4 ml) was then added as described in the "direct technic". Glucose was found totally destroyed by this procedure but solutions of inulin also showed some degradation. Solutions of inulin from 3 different sources which had similar optical densities for a given concentration when estimated by the direct technic showed varying diminution of optical densities (33%, 27%, and 17% respectively) when determined by the same technic after having been boiled in alkali. At the time we were unfamiliar with the work of Ross and Mokotoff(4) and Weil(5) who had demonstrated that commercial inulin preparations contained variable portions that were destroyed by alkali. Thus, further attempts were made to standardize the procedure by varying the strength of the alkali from 1 N to 10 N and by varying the boiling period from 2 to 10 minutes. The percentage of degradation after boiling in alkali seemed relatively constant for a single inulin source when treated under controlled conditions even though there were great variations among samples from various sources. Biological testing seemed indicated, and accordingly intravenous infusions of inulin were given to 2 patients. The urine and plasma filtrates† from these patients were analyzed for inulin by the direct anthrone technic after boiling in 5 N NaOH for 5 minutes. It was found that only 0 to 3% of the inulin in the plasma were degraded whereas 9 to 12% of the inulin in the urine behaved in similar fashion. In pursuing this problem further, samples of plasma and urine were obtained from patients undergoing inulin clearance studies in another hospital[‡] and the inulin concentrations as determined in its laboratory by the Schreiner (7) method were ascertained. The analytical data obtained using the direct anthrone technic following the treatment of the urines and plasma filtrates with NaOH were calculated relative to an inulin standard which was *not* treated with alkali. The results again demonstrated the above-mentioned finding that the inulin in the plasma seemed more resistant to alkali than the inulin in the urine. There was an average of 2.4% degradation in the former compared to 15% in urine. Plasma filtrates prepared with N/200 acetic acid gave the same results, thus ruling out the tungstic acid used in precipitation as the cause. There was poor agreement of our results relative to those obtained by the Schreiner method and this was definitely due to the use of the alkali. The inconstant distribution of the fraction of inulin which is destroyed by boiling in NaOH precludes the use of this step to determine inulin during biological studies. Specimens of blood and urine containing equal quantities of inulin but with variable percentages of labile inulin would yield erroneous total values by the use of such an analytic procedure. Young and Raisz(1) have solved this problem by preparing for biological experiments an inulin which has been made stable in alkali by pre-treatment with sodium hydroxide. This allows the subsequent use of the anthrone method for the estimation of inulin following the destruction of glucose with sodium hydroxide. An alternate anthrone method has been developed in this laboratory which entirely eliminates the need to boil in alkali and has proven to be extremely accurate.

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† Plasma filtrates prepared by addition of 0.2 ml of plasma to 2.8 ml dilute tungstic acid solution(6) in centrifuge tubes. Tubes were corked, vigorously shaken several minutes, and then centrifuged. One ml of supernatant was diluted with 1 ml of water for analysis.

Indirect anthrone technic for inulin. This technic, proposed by Seifter *et al.*(8) for the estimation of glucose and glycogen, consists of two stages: first, the suppression of the heat generated by the addition of the anthrone reagent, and next the application of external heat to produce the color reaction under controlled conditions. Experiments in this laboratory revealed that when meticulous care was taken in the first step to eliminate *all* heat during the addition of the anthrone reagent, glucose in concentrations up to 250 μg gave *no* color. This overcame the difficulty encountered with the "direct technic" where glucose gave rise to variable intensities in color during the initial contact with the anthrone reagent. Three steps were found necessary for effective suppression and instantaneous absorption of the heat when mixing the anthrone reagent with an aqueous solution; practical application was not difficult. Two ml quantities of standard aqueous solutions were introduced into matched Klett tubes which were immersed in an ice water bath. A beaker with 95% sulphuric acid and one with anthrone reagent were also chilled. After 15 minutes, 2 ml of the chilled 95% sulphuric acid were allowed to flow slowly by gravity along the inside of each Klett tube from 2 ml delivery pipettes chosen for their slow outflow rates. This preliminary dilution with sulfuric acid was necessary because icing and slow introduction was not sufficient to suppress "flash" heat production that occurred when the 95% sulfuric acid in the anthrone reagent was pipetted into a completely aqueous solution. After thorough mixing with flat bottomed stirring rods, 2 ml of chilled 0.4%[§] anthrone reagent was introduced into each tube in the manner previously employed when adding the 95% sulfuric acid. The contents of the tubes were again thoroughly stirred and the tubes were removed from the ice bath and allowed to come to room temperature. At this stage the glucose solutions had the same color as the water blank similarly treated with anthrone. In fact, this criteria served as a check as to

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[‡] We are indebted to Dr. B. Crawford of the N.Y.U.-Bellevue Medical College for plasma and urine specimens.

[§] The anthrone reagent is double the strength usually used.

TABLE I. Development of Color by Inulin and Glucose Solutions at Varying Temperatures following Mixing with Anthrone Reagent by a Technic Which Had Suppressed Color Formation before Heating.

Heat and temp.		Time, min.	Klett-Summerson readings (at λ 620) Glucose 100 γ	200 γ	Inulin 10 γ	20 γ
Room temp.		30	.25	.0	13.3	24.5
Incubator	37°C	60	.0	.1	19.0	37.5
Water bath	40	30	1.3	2.5	32.0	61.0
Idem	50	20	5.0	10.0	37.5	74.0
"	56	10	4.5	9.1	37.2	74.5

Results of Analyses of Combinations of Glucose and Inulin (Color Developed after 10 Min. in Water Bath at 56°C). These are compared with the sum of results obtained separately for each carbohydrate.

γ glucose	γ inulin	Klett-Summerson readings Determined value	Calculated value
100	10	43.0	41.7
100	20	79.8	79.0
200	10	46.5	46.3
200	20	83.0	83.6

whether the proper technic was used. A slight color was usually noted at this stage with inulin samples but did not influence later results.

Subsequent heating gave reproducible and consistent color formation with both inulin and glucose solutions. Representative data obtained at various levels of heating are given in Table I. Young and Raisz(1) found that heating at 75°C for 5 minutes led to maximal development of color on the part of inulin. Since the aim of the present method was to suppress formation of color by glucose, lower temperatures were selected. At 56°C the color formation with inulin was found to reach a maximal plateau level in 10 minutes. Since most clinical laboratories have a water bath for serological work available at this temperature, no special equipment is necessary. Thus, after the above preparations were removed from the ice water bath and after they reached room temperature, they were put into the water bath at 56°C for exactly 10 minutes. Fig. II gives the results obtained by this procedure with standard solutions of glucose and inulin. The color developed by inulin was linear to concentrations up to 25 μg.

Color formation by inulin was 83 times greater than that produced by an equivalent amount of glucose. Most important were the findings that the reaction on the part of glucose was constant and also that the color produced by glucose was additive to that produced by inulin. The observed analytic values of combinations of inulin and glucose corresponded closely to the calculated values as shown in the bottom of Table I. The recovery of unknown quantities of inulin added to varying known quantities of glucose has always been better than 98%. These facts permitted the use of a chromogenic blank for corrections in the analyses of inulin in blood and urine as has been done in other chemical procedures for inulin.

The method has been successfully applied for the micro-analysis of inulin in blood and urine using tungstic acid filtrates for plasma. Excellent agreement has been found with other chemical methods. This will be reported at the completion of our present work to allow the determination of both inulin and

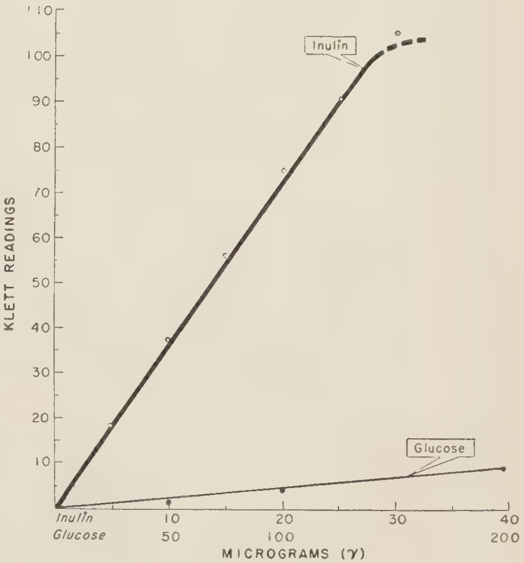

FIG. 2. Color formation by inulin and glucose with the "Indirect Technic." Color formation by inulin and glucose after heating with anthrone reagent in water bath 56°C for 10 min. Initial mixing of solutions with anthrone reagent was accomplished by eliminating significant heat of solution. Klett readings (λ = 620 μ) are given.

glucose in the same specimen of capillary finger blood by means of a chemical procedure similar to that reported here.

Summary. Use of the anthrone reagent offers a sensitive and accurate method that can be applied for the estimation of microquantities of inulin. Steps are given to prevent interference by glucose through the means of controlling the temperature of reaction. Boiling in alkali cannot be used to eliminate glucose because of an irregular distribution between blood and urine of the alkali labile fraction of inulin.

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Comparison of Blood Cell Counts from Major Vessels in the Dog.* (21098)

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Some investigators(1-3) have reported impressive differences in the number of cells found in blood taken from various sources in laboratory animals. However, other workers (4-6) have failed to confirm this observation. The present investigation was carried out on both normal and splenectomized dogs. Comparisons were made between samples obtained simultaneously from the aorta, hepatic vein, caudal vena cava, superior vena cava, and/or right ventricle in unanesthetized dogs and dogs anesthetized with pentobarbital sodium.

Methods. The catheterization of major arterial and venous vessels was performed in unanesthetized dogs by inserting small polyethylene catheters through a 15-gauge needle placed in the vessel after procaine infiltration of the area. The catheter was positioned by fluoroscopic visualization, the stylet removed and the catheter filled with 0.5% heparin in sterile saline. Catheters were placed in the aorta and in either the right ventricle or the superior vena cava. In addition to the comparisons with unanesthetized animals, a series of experiments was performed on animals under pentobarbital anesthesia (30 mg/kg body weight). In these experiments the catheters were placed in the right ventricle, and in the aorta and caudal vena cava at the anterior margin of the pelvic girdle. Additional data were accumulated from experiments in which Cournand catheters were placed in various positions in anesthetized and heparinized (5 mg/kg) animals, and in which all the catheters were introduced by exposing and ligating the vessels. In this series, comparisons were made with samples from the aorta and hepatic vein, and from the right ventricle and peripheral or hepatic vein. For the comparisons reported in this investigation data from samples drawn after any experimental procedure beyond that concerned with placing the catheters have not been included. The erythrocyte and leukocyte counts were done in duplicate, using National Bureau of Standard certified pipettes. The duplicate counts were required to check within 10%. At least 200 cells were counted in the differentials and checks were required between the first and second halves of the counts. Lymphocytes and monocytes were grouped together as mononuclear cells and no differentiation was made as to nuclear configuration of neutrophiles.

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TABLE I. Comparison of Blood Cell Distribution in Normal and Splenectomized Dogs in Anesthetized and Unanesthetized Dogs. W = total leukocytes, R = erythrocytes, E = eosinophiles, M = mononuclear cells, N = neutrophiles, S. E. = stand. error.

Effect of splenectomy															
Comparison	Aorta vs caudal vena cava					Right ventricle vs caudal vena cava					Aorta vs right ventricle				
No. of comparisons	26					26					37				
Cell type	W	R	E	M	N	W	R	E	M	N	W	R	E	M	N
$\bar{x}_n - \bar{x}_s$ *	106	142	53	71	135	130	88	309	131	295	248	16	16	636	262
S. E.	174	80	98	133	172	210	130	195	170	221	494	220	413	562	1064
"t" value	.60	1.78	.54	.53	.78	.62	.68	1.58	.77	1.33	.50	.07	.04	1.13	.24

Effect of pentobarbital										
Comparison	Aorta vs 'unmixed venous'					Aorta vs right ventricle				
No. of comparisons	26					37				
Cell type	W	R	E	M	N	W	R	E	M	N
$\bar{x}_u - \bar{x}_a$	113	20	367	618	199	290	27	163	628	386
S. E.	308	170	247	329	523	495	190	413	562	1066
"t" value	.37	.12	1.48	1.88	.38	.58	.14	.39	1.12	.36

* Leukocyte difference in cells/mm^3, erythrocyte difference in thousands of cells/mm^3.

Results. Statistical analyses were performed on the difference in the distribution pattern of blood cells in the anesthetized and unanesthetized animals. Student "t" values were calculated for the differences in the comparisons of leukocytes, erythrocytes, eosinophiles, mononuclear cells and neutrophiles, and in all cases the levels of significance were above 5% (Table I). Similar comparisons were made for the relationship between the distribution of blood cells in normal and splenectomized dogs. No significant difference (at the 5% level of significance) was observed in any blood cell type when normal and splenectomized dogs were compared (Table I). Thus, statistically significant differences did not exist in experiments from anesthetized and unanesthetized animals, nor in experiments from normal and splenectomized dogs. The experiments, therefore, could be grouped with reference only to the source of the samples, in so far as a study of the comparisons of blood cell counts from various regions was concerned.

Studies were made of the cellular distribution between the arterial and venous sides of the circulation. Comparisons were made between the aorta and hepatic vein, aorta and superior vena cava, aorta and caudal vena cava, and the aorta and right ventricle. The studies demonstrated no significant differences in the erythrocytes nor in any leukocyte cell type from arterial and venous samples drawn simultaneously (Table II). Additional studies were carried out on the distribution of circulating blood cells in the major venous vessels. They consisted of cell count comparisons between blood withdrawn simultaneously from the right ventricle and hepatic vein, peripheral vein and hepatic vein, right ventricle and caudal vena cava, and the right ventricle and peripheral vein. No significant differences were demonstrated in any blood cell type (Table II).

Discussion. One of the primary considerations in the distribution of blood cells within the vascular system is that the sample should be a valid representation of blood flowing in the vessels. A blood sample obtained by puncturing a large vein probably gives a reliable estimate of the cell content in that vein.

TABLE II. Comparisons of Blood Cell Counts in Samples Drawn Simultaneously from 2 Major Vessels in Dogs. W = total leukocytes, R = erythrocytes, E = eosinophiles, M = mononuclear cells, N = neutrophiles.

Arterial and venous comparisons										
Comparison	Aorta vs hepatic vein					Aorta vs right ventricle				
No. of comparisons	18					40				
Cell type	W	R	E	M	N	W	R	E	M	N
Mean diff.* $\frac{\Sigma(A-B)}{n}$	223	176	18	64	213	113	6	13	13	41
"t" value	1.20	1.18	.64	1.28	1.17	1.02	1.17	.13	.05	.36
Comparison	Aorta vs superior vena cava					Aorta vs caudal vena cava				
No. of comparisons	10					29				
Cell type	W	R	E	M	N	W	R	E	M	N
Mean diff.* $\frac{\Sigma(A-B)}{n}$	178	190	324	614	348	47	109	43	9	146
"t" value	.70	1.31	1.42	2.04	.71	.71	1.81	1.07	.19	1.66
Comparisons of venous samples from different sources										
Comparison	Right ventricle vs hepatic vein					Right ventricle vs caudal vena cava				
No. of comparisons	7					29				
Cell type	W	R	E	M	N	W	R	E	M	N
Mean diff.* $\frac{\Sigma(A-B)}{n}$	334	13	27	152	148	58	80	123	63	91
"t" value	.79	.10	.30	1.37	.24	.64	1.43	1.17	.91	.92
Comparison	Right ventricle vs peripheral vein					Peripheral vein vs hepatic vein				
No. of comparisons	7					7				
Cell type	W	R	E	M	N	W	R	E	M	N
Mean diff.* $\frac{\Sigma(A-B)}{n}$	1347	197	28	26	1177	1009	318	55	120	1028
"t" value	1.66	1.89	.22	.22	1.58	.94	1.54	.58	.97	.93

* Leukocyte difference in cells/mm^3, erythrocyte difference in thousands of cells/mm^3.

However, investigations carried out on blood drawn from punctures of the skin, commonly referred to as capillary blood, may be unreliable unless sufficient precautions are instituted to insure that misinterpretations are not made. Another important consideration in studies of the intravascular distribution of blood cells is that comparisons should be made only on samples drawn under the same experimental conditions. If direct comparisons are to be made, the samples should be drawn simultaneously.

Investigations of the distribution of erythrocytes have suggested that arterial and venous or peripheral blood have the same circulating erythrocyte content(1,6,9). One study(3) described experiments on the guinea pig which were interpreted as indicating a lower erythrocyte level in the heart than in the periphery. However, the experimental procedure consisted of collecting peripheral blood from a nick in the ear, then anesthetizing the animals with pentobarbital and collecting heart blood by direct punctures. It has been demonstrated(7,8) that pentobarbital administration results in an erythrocyte decrease of approximately the same magnitude as the difference reported in that study. Because of the design of their experiment, it would appear that those workers measured the effect of the anesthetic agent on the erythrocyte count and interpreted it as an effect of comparing the 2 sampling sites. The experiments being reported indicated that no erythrocyte count differences were found when arterial and venous blood samples were collected simultaneously. The data also suggested that there were no differences in various parts of the venous system, and that neither splenectomy nor anesthetization with pentobarbital affected the distribution of circulating erythrocytes in the major vessels of the dog.

Most of the disagreement regarding the distribution of blood cells is concerned with the leukocyte count. A difference was reported for tail blood and heart blood on rats which were stunned by a blow on the head after collecting the sample from the tail and before the heart blood was collected(1). The animals were not in comparable experimental conditions at the time the samples were collected. When these workers(2) compared the results of blood counts drawn simultaneously from anesthetized animals, they found no difference following ether anesthesia, while there was a significantly lower leukocyte count in blood from the heart than from the tail when pentobarbital was used as the anesthetic agent. They also reported a significant difference in unanesthetized rats which were immobilized while a cardiac puncture was performed. Other investigators(5) demonstrated no difference between tail and heart blood from rats anesthetized with ether or from rats killed with a blow on the head. Blood from the left heart, periphery or venous drainage in unanesthetized dogs showed no significant cell count differences(6). In an excellent study of the distribution of leukocytes in the vascular system, the importance of correctly interpreting results from peripheral blood samples was demonstrated(10). It was shown that the first drop of blood from a lancet-puncture had a significantly higher leukocyte count than the succeeding blood. Only by conscientiously attempting to collect the first drop of blood flowing from the puncture was it possible to demonstrate a leukocyte count different from that in the major vessels. It was suggested that the increased leukocyte count seen in the first drop of blood does not represent the leukocyte concentration in the capillary blood, but reflects a flushing out of leukocytes which have adhered to the endothelium of the para-capillary veins. This observation may account for some of the reports that peripheral blood has a higher leukocyte count that heart blood.

Summary. Blood cell counts were compared in samples drawn simultaneously from the aorta, right ventricle, superior vena cava, caudal vena cava, hepatic vein, and/or peripheral veins in dogs. The study was done on normal and splenectomized dogs, either unanesthetized or anesthetized with pentobarbital. No differences in erythrocyte or leukocyte distributions were demonstrated as a result of anesthetization or splenectomy. The erythrocyte and leukocyte counts were the same in arterial and venous blood samples and in venous samples from different major vessels. Some factors are discussed which may be responsible for apparently contradictory reports concerning cell count distribution in circulating blood.

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Retardation of Growth of Implants of Carcinoma 755 in Cortisone-Injected Mice. (21099)

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Heilman and Kendall(1) reported that 17-hydrocorticosterone prevented the growth of implanted undifferentiated lymphosarcoma in mice. Earlier, Murphy and Sturm(2,3) noted a similar adrenocortical control of lymphoid tumors. Wooley(4) reported that cortisone reduced the rate of growth of lymphoid tumors when given orally, and reduced less when administered subcutaneously. Gottschalk and Grollman(5) reported the inhibition of a transplanted mouse mammary carcinoma by large doses of cortisone, and Baserga and Shubik(6) showed that the growth rate of a transplanted mouse adenocarcinoma was temporarily inhibited by the administration of cortisone. It was of interest to investigate the effect of cortisone* on growth of a transplantable epithelial tumor, carcinoma 755 in C57 mice and to study the mechanism of its action.

Method. Approximately 200 mice, 4-5 months of age, of the C57 strain inbred for many generations were used in these experiments. Animals in a single experiment received implants in the axilla, by trocar, of approximately equivalent fragments of carcinoma 755 from a single, fresh, dissected tumor free of necrosis. One group of the tumor implanted mice were injected subcutaneously once weekly with 2.5 mg cortisone in 0.1 cc vehicle starting at various periods after transplantation. Another group received 0.1 cc of the vehicle without cortisone or 0.1 cc saline weekly and some received no additional treatment after tumor transplantation. The tumor growth was followed daily by palpation and measurement of the tumor. The animals were sacrificed at intervals, complete autopsies were performed, the tumors weighed, and tissue examined histologically.

Results. A) *Growth of tumor.* Fig. 1 is a composite of a representative experiment in which the mice received 2.5 mg cortisone once weekly, starting 24 hours after transplantation. Controls received 0.1 cc saline or 0.1 cc cortisone vehicle. The mice were sacrificed 28 days later. Each vertical column represents weight of tumor from one mouse. The average tumor weight in the saline control was 3.14 g, in the vehicle control 3.02 g, and in the cortisone-treated mice 0.39 g. In the saline controls the tumor became palpable 13 days after implantation (average), in the vehicle controls 13.3 days, and in the cortisone-treated after 19.9 days. A similar result was obtained in female mice in which the first cortisone injection was administered 6 days after tumor implantation. These animals were sacrificed 33 days later. The tumors from 7 cortisone-treated animals averaged 0.764 g, while those of 8 untreated controls averaged 5.74 g.

B) *Tissue studies.* 1) *Gross appearance.* The tumor fragment in control mouse is seen distinctly within the formalin fixed subcutaneous tissue while in the cortisone treated mouse it is difficult to identify the tumor particle during the first 2 weeks because it is sur-

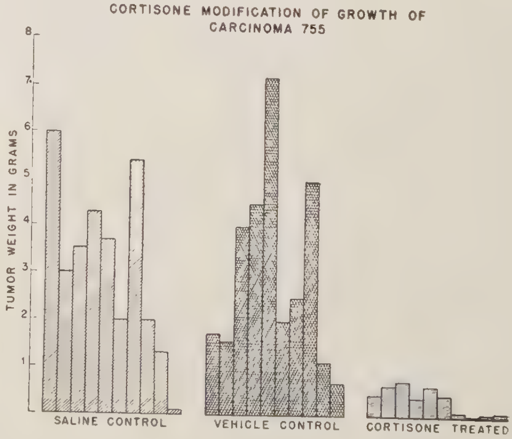

FIG. 1.

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* Cortagen—Schering.

rounded by a dense, opalescent, mucoid fluid.

2) *Histology.* In the untreated mouse a moderate number of polymorphonuclear leukocytes are present about the trocar tract. In 72 hours new capillary channels begin to appear in the tissue surrounding the tumor. An occasional short, isolated strand of degenerate collagen is seen about the trocar tract. In 4-7 days, buds of the tumor grow into the adjacent areolar tissue. In 10 days there is considerable invasion by the tumor into the surrounding tissue.

In the cortisone treated mouse there are fewer polymorphonuclear leukocytes about the tumor than in the control. A discontinuous intensely eosinophilic membrane resembling "degenerate" or "necrotic collagen" is present in the tissue adjacent to the site of the tumor fragment and to the tract made by the trocar. (In the controls this is only occasionally seen and is considerably less in amount than in the cortisone-treated animals.) Only a few polymorphonuclear leukocytes are present about this tract. In the first week, very few if any new capillaries are seen in the tissues about the tumor implant. Fluid often appears in the blind end of the trocar tract. The tumor, rather than budding into and invading the adjacent tissues, seems to progress along the surface of the sinus tract and extend centripetally into the accumulated fluid as though growing into a tissue culture medium. This growth is slight. At this stage tumor growth is not seen in the areas lined by the eosinophilic membrane. Budding of the tumor into adjacent tissue is not evident until after 2 weeks.

Discussion. In studying the effect of various substances on transplantable tumors it is necessary to consider 1) the survival of the tumor transplant and retention of its growth potentialities before the establishment of a blood supply from the host; 2) the effect of the substance on the host tissues, particularly as it effects the immediate milieu of the tumor and the establishment of a blood supply, and 3) the response of the transplant after a blood supply from the host has been established. In the case of cortisone administered in the first few days after transplanting carcinoma 755, the tumor remains viable but a strong inhibiting effect on growth of the tumor occurs. Histological studies show that during this period cortisone has a profound effect on the immediate environment of the tumor transplant. Fluid often appears in the blind end of the trocar tract, and the tumor, rather than bud into the surrounding tissue, seems to extend along the surface of the sinus tract skipping for the most part the areas lined by the membrane of "degenerate collagen." No new capillaries are seen in the first week after tumor transplantation. These environmental changes in the host produced by cortisone, administered in the first days after transplantation, could account for the cortisone effect on the tumor, *i.e.*, retardation in growth due to altered milieu and delay in capillary formation. This latter effect is not entirely unexpected since it has been demonstrated that cortisone inhibits new vessel formation in granulation tissue(7-12). Furthermore, the formation of the eosinophilic membrane about the trocar tract appears to be unfavorable to growth and spread of the tumor. This may be considered as the result of an exaggerated reaction of the collagen to the trauma of the trocar, and would correspond to the pronounced degenerative changes produced by noxious agents in animals receiving cortisone (13-16). Viability of the transplant during this stage is probably dependent upon nourishment from the fluid adjacent to the tumor. Once an ample capillary bed is formed, providing a blood supply from the host, the tumor starts to grow and the inhibitory effect of cortisone is abolished. In the later stages of tumor growth, cortisone does not exert an inhibitory effect but in some instances there was a tendency to enhance tumor growth after cortisone administration.

In this communication we did not include the effect of cortisone on permeability of the host's capillaries, particularly in the period immediately following tumor implantation (17,18).

Since infections are not infrequently encountered in cortisone-treated mice(13) and cortisone produced visceral changes which, in some respects, are similar to those caused by inanition(14), these factors must be eliminated as contributing elements in the carcinostasis. In our experiments smears and cultures were taken from liver, kidney, and tumor of cortisone-treated mice, particularly in those in which the tumor growth was inhibited. With very few exceptions no bacteria were found on smears or in cultures. Because of this, infection as a contributory factor was ruled out. The fact that the animals appeared well nourished and continued to gain weight spoke against inanition as a factor in producing the retardation of tumor growth.

Summary. 1. Cortisone, when administered during the first few days after transplantation of carcinoma 755 in C57 mice, has a profound inhibitory effect on growth of the tumor but the tumor remains viable. 2. These effects of cortisone appear to be due to the change in the environment and to the delay in new blood vessel formation about the tumor transplant.

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Accumulation of Astatine^{211} by Thyroid Gland in Man.* (21100)

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Astatine^{211}, which is the last member of the halogen group of elements, has been found to be accumulated by the thyroid glands of experimental animals to a degree similar to that of iodine^{131}(1-4). Since the average energy of the alpha-particles arising from the disintegration of At^{211} is 6.8 Mev and the range in tissue is but 70μ, the degree of specific ionization produced as compared to the beta-particles from I^{131} is much greater. The maximum energy of I^{131} beta-particles is 0.6 Mev and the range is 2,000 μ. The motivation for these extensive studies with At^{211} has been the consideration that the short range and high specific ionization of the alpha-particles of At^{211} as compared to the much longer range and more weakly ionizing beta-particles from I^{131} might have clinical applications in treatment of thyroid disorders, and in particular hyperthyroidism.

For several years this laboratory has been engaged in a study of the rate and degree of accumulation of both I^{131} and At^{211} in the thyroid gland and other tissues and organs of experimental animals. The acute and chronic effects of relatively large amounts of these two radiohalogens has also been investigated(2-4). The results of these experiments

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* This work was performed under contract of the University of California under the U. S. Atomic Energy Commission.

TABLE I. Uptake of Orally Administered Astatine^{211} (EKA-Iodine) by Human Thyroid Gland in Various Disorders of That Organ; 8 Patients.

Age	Sex	Thyroid sample	Diagnosis	Time, hr	% At^{211} in sample	Conc. At^{211}, %/g wet tissue	mg I_2 in sample	mg I_2/g wet tissue
∼40	♀	Left L*	Grossly normal	15	5.95	.54	32	2.94
		Right L	” ”	18	11.85	1.19	30.1	3.01
		Cervical lymph node	Papillary adenocarcinoma	18	$<$.01	$<$.001	—	—
41	♀	Right L	Non-toxic metaplasia	13¼	2.85	.27	5.19	.49
31	♀	Whole G	Non-toxic nodular goiter with cyst formation	16¼	6.75	.55	6.34	.53
35	♀	Whole G	Toxic goiter (Lugolized)	15	6.66	.21	13.42	.43
52	♂	Right L	Graves' disease (thyroxine, Lugolized)	18	.172	.017	5.8	.58
43	♀	Left L	Non-toxic nodular goiter	14	6.55	.26	—	—
56	♂	Left L	Grossly normal	13½	4.90	.39	3.78	.50
		Right L	Non-toxic nodular goiter	13½	2.56	.024	7.12	.07
68	♂	Whole G	Non-toxic nodular goiter	22	4.61	.021	28.5	.13

* L = lobe; G = gland.

indicated that it would be safe to attempt tracer studies with At^{211} in patients that were to be subjected to surgical thyroidectomy.

Methods. The At^{211} was prepared by a modification of the procedure described by Garrison, *et al.*(5). Each patient received 50 microcuries of At^{211} in a solution of 25 cc of water at intervals which ranged from 13¼ to 22 hours prior to surgery. Food was withheld for at least 2 hours prior to and after the oral administration of the solution of At^{211}. The patients selected in this group were all more than 30 years of age and in the majority of instances, either a complete thyroidectomy was performed or a remaining lobe of the thyroid gland was removed. The series of 8 patients included one with a papillary adenocarcinoma of the thyroid gland with metastases to the cervical lymph nodes. Following removal, the surgical specimen was immediately weighed and a weighed portion was taken for routine hospital pathological studies while the remainder was brought to this laboratory for the assay of its At^{211} content as well as the total iodine content. Before the specimen was to be subjected to physical and chemical processing, a small representative portion was removed by the use of the dissecting microscope. This specimen was then fixed in 10% neutral formalin, subjected to routine paraffin impregnation and clearing technics, and finally stained with hematoxilin and eosin. Since the decay scheme of A^{211} is associated with the emission of 80 Kev x-rays(6) it was possible to make a relatively accurate measurement of the At^{211} content of the wet sample employing a sodium-iodide thallium iodide activated crystal scintillation counter. Two patients had previously received I^{131} which made this type of determination essentially impossible due to the presence of gamma-rays from I^{131}. The stable iodine content in these studies was determined in a carbon tetrachloride solution by means of a spectrophotometer rather than by titration employing dilute solutions of sodium thiosulfate used in earlier experiments (7). The At^{211} was quantitatively isolated from the specimens by a procedure to be reported elsewhere(8).

Results. The results of the experiments are shown in Table I. A number of observations may be made. The accumulation of At^{211} by the thyroid gland appears to be relatively higher than has been observed in some of the previously reported experiments which employed laboratory animals(2). Admittedly, the number of patients is too limited to draw conclusions of a statistical nature, but there certainly does appear to be a trend of a relatively higher uptake. It should be noted that in 3 patients only one lobe of the thyroid gland was available for assay. Moreover, histologically, the thyroid tissue obtained from most of these patients was obviously quite abnormal. A very low uptake of At^{211} was noted in a patient that had had a very unusual series of therapeutic attempts to control his disease and at one stage he had received both thyroxin and Lugol's Solution. In the one patient from whom an entire relatively normal thyroid gland was available, the total uptake was approximately 17% of the administered dose. There appears to be a correlation of the amount of At^{211} accumulated per gram of tissue with the stable iodine content per gram of tissue. In general, when there was a relatively high total iodine content, the astatine content was increased. Finally it should be noted that in the patient with a metastatic papillary adenocarcinoma there was no demonstrable accumulation of At^{211} in the cervical lymph nodes which contained essentially nothing but neoplastic tissue showing no tendency towards the formation of either follicles or colloid.

Discussion. With the exception of the one patient who had received thyroxin, there was quite a good correlation between accumulation of At^{211} per gram of thyroid tissue and the amount of stable iodine present. Attempts to correlate the histological findings in thyroid tissue from the 8 patients with the amount of stable iodine present per gram and the uptake of At^{211} is difficult. The interpretation of the apparent relatively greater concentration in At^{211} by human thyroid tissue as contrasted to animal studies is hard to establish. If this apparent effect can still be demonstrated involving a larger number of patients, then consideration must be given to the factors such as total iodine intake and the possible existence of the species difference. The At^{211} uptake studies in the monkey are too limited to be of any value in attempting to make any comparison with humans presented in this paper (2).

The evaluation of the potential use of At^{211} for the therapy of human thyrotoxicosis must of necessity await much more information derived from both further studies with experimental animals and more extensive human uptake studies.

The chemical properties of astatine are considerably different from those of iodine in many respects(9). An indication of the biochemical differences between astatine and iodine have been presented in another paper. The effect of pre-treatment with propyl thiouracil resulted in the enhancement in the accumulation of At^{211} by a factor of nearly 20 in the thyroid gland in the rat while there was at the same time, a decrease of accumulation of I^{131}(10). With these considerations in mind there is a very remote possibility that certain types of neoplasms of the thyroid gland may more effectively accumulate At^{211} than I^{131}. This concept is purely hypothetical and is based upon the probability that At^- may be oxidized to At^o more readily than I^- might be oxidized to I_2. It should be emphasized that this phase of the discussion is purely speculative in nature and the very questionable validity of this theory can only be tested by doing tracer studies on many patients suffering from various types of neoplasms of the thyroid gland.

Summary. 1. Accumulation of At^{211} by thyroid gland in patients suffering from various disorders of that organ has been demonstrated. 2. Accumulation of At^{211} by thyroid glands of these patients appears to be relatively higher than has been observed in experiments employing rats. 3. There may be a correlation between the uptake of At^{211} and stable iodine in thyroid tissue. 4. One patient with papillary adenocarcinoma showed no discernible accumulation of At^{211} in metastases present in cervical lymph nodes.

Production of At^{211} was the responsibility of Mr. G. Bernard Rossi and the staff of the 60-inch cyclotron at this laboratory. The continued interest and encouragement by Dr. Robert S. Stone, Dr. Earl R. Miller, the Staff of the Division of Radiology; Dr. Theodore L. Althausen, Dr. Morris E. Dailey, Dr. H. Glenn Bell, Dr. Henry H. Searls, and the surgical staff at the Medical School made the experiments with human subjects possible. The cooperation of members of the Division of Pathology was most helpful. Dr. William A. Reilly, of the Medical School, and Director of the Isotope Unit at the Veterans Administration Hospital at Fort Miley made available the use of their facilities for one of the human studies.

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Effect of Pretreatment with Propyl Thiouracil on Accumulation of Astatine^{211} by Thyroid Gland of the Rat.* (21101)

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It has long been known that a variety of chemical agents act as anti-thyroid drugs to reduce or almost completely prevent the introduction of iodine into tyrosine and subsequently the production of thyroid hormone (1,2). Propyl thiouracil and related antithyroid drugs have been used extensively in the treatment of human thyrotoxicosis(3,4). Astatine is the heaviest member of the halogen group, and certain of its chemical properties resemble those of iodine. There are, however, some rather significant dissimilarities(5). Although 18 radioactive isotopes of astatine have been identified, no stable isotopes of this element are known to exist(6). The particular radioisotope used in these and other studies is the 7.3 hour At^{211} which decays by the emission of alpha-particles and 80 Kev x-rays.†

The capacity of the thyroid gland to concentrate and retain At^{211} has been demonstrated in experimental animals and man (7-10). The present studies were undertaken in order to determine the possible effect of propyl thiouracil upon accumulation and retention of At^{211} by the thyroid gland of the rat and to compare this effect with that obtained using carrier-free I^{131}. It was also hoped that these experiments would help to shed some light on the mode of entry of astatine into the thyroid gland and on its chemical state in that organ.

Methods. The animals employed for these studies were 75-day-old female Sprague-Dawley rats weighing about 165 g, all received from the dealer at the same time and maintained for 3 weeks prior to the start of the experiment on tap water and a pelleted stock diet which is in general use throughout the University of California Radiation Laboratory.‡ Both food and water were given

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* This work was performed under contract of the University of California under the U. S. Atomic Energy Commission.

† Sixty per cent of the At^{211} nuclei decays by K-capture to form Po^{211} with a 0.5 second half-life, and 40% decays by alpha-particle emission to form Bi^{207}.

‡ This diet is similar in composition to "Diet 14" developed by the University of California Institute of Experimental Biology and contains a fairly large amount of added iodide. To every 270 lb of feed is added 300 ml of an 0.45% solution of KI.

TABLE I. Effect of Administration of 0.1% Propyl Thiouracil in Drinking Water for 11 Days on Body Weight, Thyroid Weight and Thyroidal Uptake of Intravenously Administered At^{211} and I^{131} in the Female Sprague-Dawley Rat.*

	Propyl thiouracil water	P†	Tap water
Animal wt	156.4 ± 1.68 g	$<.02$	164.8 ± 2.88 g
Thyroid "	37.4 ± 1.31 mg	$<.01$	15.2 ± .61 mg
Thyroid uptake, % admin. At^{211}	3.82 ± .26	$<.01$	.23 ± .0025
Thyroid conc., % At^{211}/g wet tissue	105.4 ± 6.61	$<.01$	16.2 ± 1.8
Thyroid uptake, % admin. I^{131}	1.13 ± .16	$<.01$	7.28 ± 1.47
Thyroid conc., % I^{131}/g wet tissue	30.3 ± 4.5	$<.01$	445 ± 83.3

* Stand. error of mean $= \sqrt{\dfrac{\Sigma d^2}{n\,(n-1)}}$.

† For calculation of P see Fisher(12).

ad lib. Propyl thiouracil was administered to 20 rats in the drinking water at a concentration of 0.1% by weight for 11 days. On the 10th day, 10 of these rats were each given 5 μc of At^{211}§, and 10 were each given 6 μc of I^{131}. Both At^{211} and I^{131} were administered intravenously. Ten rats that had received no pretreatment served as controls. Five of these were each given 5 μc of At^{211} and 5 each received 6 μc of I^{131}. Twenty-one hours after the administration of the radiohalogens the rats were sacrificed with chloroform and the thyroids were dissected out and weighed. The At^{211} and I^{131} contents of the thyroid glands were measured with an NaI-TlI scintillating crystal gamma counter. It was possible to measure the At^{211} in this manner, since its radioactive decay is associated with an 80 Kev x-ray.

Results. The results of these experiments are shown in Table I. The single most impressive observation is the nearly 20-fold enhancement of the accumulation of At^{211} in the thyroid glands of the animals treated with propyl thiouracil. This is in distinct contrast to the diminution of the accumulation of I^{131} in the thyroid glands of the animals treated with this drug, a result that has been shown previously by Taurog *et al.*(1). In fact, the concentration of At^{211} by the thyroid glands of the treated animals is more than 3 times the concentration of I^{131} in this group.

It should be noted that the values for the uptake of both I^{131} and At^{211} in the thyroid glands of the control animals are somewhat low. This is presumably due to the presence of a relatively large amount of stable iodine in the diet employed. It has been shown that the administration of stable iodide decreases the accumulation of both I^{131} and At^{211} in the thyroid gland of the rat(8). The uptake of At^{211} in the treated rats is far greater than has been seen previously even when they had been maintained on a low iodine diet(8).

Discussion. These results were quite unexpected. A possible explanation for the tremendous increase in the thyroidal uptake of At^{211} following the administration of propyl thiouracil is presented here with the understanding that it is highly speculative.

The apparent action of propyl thiouracil and related anti-thyroid drugs is to prevent the synthesis of iodotyrosine and similar compounds(1). From the results of experiments described here it appears that the effect of propyl thiouracil on the accumulation of At^{211} by the thyroid gland is quite different from its effect on the uptake of I^{131}. The inorganic chemistry of astatine is complex and not too well understood and nothing is known about its biochemistry. Astatine has been shown to possess at least 4 valance states, At^-, At^o, AtO^- and a higher oxidized state(5). It is likely that in the thyroid gland there are enzymes capable of oxidizing At^o to AtO^-, the lowest of the oxidized states, and will thus tend to rob the thyroid gland of its accumulated astatine. In the presence of propyl thiouracil it may very well be that the oxidation of At° to AtO^- is prevented permitting the continued accumulation and retention of astatine in some type of loose organic binding.

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§ The physical and chemical procedures for the preparation of At^{211} are given elsewhere(11).

Summary. A study has been made of the accumulation of I^{131} and At^{211} in normal and propyl thiouracil-treated rats. A very marked enhancement of the accumulation of At^{211} in the thyriod gland has been observed following administration of propyl thiouracil. This is in contrast to the diminution of the uptake of I^{131} by the thyroid glands of the rats receiving propyl thiouracil.

The authors wish to thank Dr. Warren M. Garrison for his continued interest in this work and his many helpful suggestions, Mr. G. B. Rossi and the crew of the 60-inch cyclotron who were responsible for the production of the astatine, and Mrs. Marilyn Williams for technical assistance.

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Localization of Antimony in Blood. (21102)

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This study is an outgrowth of an observation by Andrews and Hamilton of this laboratory. Rats to which they gave a single intravenous injection of fuadin labeled with Sb 122, 124 showed a relatively high concentration of the antimony in the erythrocytes. The concentration tended to be maximal in approximately 8 days. Earlier workers had noted high concentration of antimony in red cells following administration of tartar emetic and other antimonial drugs(1,2). Several alternative hypotheses, which might be offered to account for these observations, have been explored. The results obtained are consistent with the hypothesis that the antimony in the red cells is attached to the protein portion of the hemoglobin.

Methods. The labeled antimony catechol disulfonate complex (fuadin) for this work was prepared as follows: A quartz ampoule containing 75 mg of Sb_2O_3 was irradiated in the Oak Ridge pile 1 week or more. The ampoule was transferred to a holder behind a lead screen, the neck was broken off, and 0.5 ml of concentrated HCl was introduced by means of a pipette. The mixture was stirred 10 minutes by a slow stream of air bubbles from a capillary tube. A solution of 0.63 g of sodium catechol disulfonate[†] in a little water was added while stirring was continued. These quantities provided an excess of the complexing agent. The solution was washed into a graduated test tube, neutralized with 10% NaOH to pH 7, and was diluted to 25 ml with water.

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[†] We are indebted to Dr. C. M. Suter of Sterling-Winthrop Research Institute for supplying this compound.

Results. The apparent increase of the relative concentration of antimony in the red cell fraction with time up to 8 days suggested that the antimony found in the red cells may have entered during their development. The following experiments, however, show that this is not the only way in which antimony can enter the red cell fraction. Two ml of a dilute solution of labeled fuadin in 0.9% NaCl, 10 ml of 0.9% NaCl, and 1 ml of whole, fresh, unheparinized rat blood were shaken 3 minutes. After standing 45 minutes at room temperature, the mixture was centrifuged 30 minutes. The counting of an aliquot portion of the clear supernatant liquid showed that over 10% of the antimony had been removed beyond the effect of mere dilution. It seemed possible that reaction of the antimony with the plasma protein might have formed an insoluble precipitate, which was thrown down by centrifuging. However, a 4.7 ml portion of the plasma was mixed with 5.4 ml of 0.9% NaCl and 2.0 ml of the dilute fuadin solution, allowed to stand 30 minutes, centrifuged and filtered. An aliquot portion of the clear filtrate showed only 2% reduction in counts per minute, which was well within the limit of error of the counting equipment.

The next hypothesis examined was that the antimony is removed from the solution by becoming adsorbed on the surface of the red cells. Various quantities of a suspension of repeatedly washed rat red blood cells were mixed with 2.0 ml portions of the fuadin solution, diluted to 11 ml with 0.9% NaCl, shaken 15 minutes, and centrifuged 20 minutes. The results shown in Table I indicate that, although there may have been some removal of antimony by the cells, it was not so rapid as would be expected for surface adsorption.

Another experiment demonstrated that removal of antimony by the red cells is marked when more time is allowed. In each of 2 centrifuge tubes, 3.0 ml of defibrinated rat blood was mixed with 10 ml of 0.9% NaCl containing a trace of fuadin and was allowed to stand at room temperature with occasional gentle agitation. In the tube that was centrifuged at the end of 2 hours the ratio of the counts per minute per ml of cells to the counts per minute per ml of supernatant liquid was 4.9. At the end of 6.7 hours the ratio was 6.2. These results were taken as an indication that the antimony penetrates the cells and becomes attached at a moderate rate of speed.

TABLE I. Removal of Antimony from Fuadin Solution by Short Contact with Red Cells of the Rat.

Quantity of cell suspension, ml	c/min.
.0	3531
.1	3520
.65	3305
1.6	3356
6.0	3343

A 386 g white, male Wistar rat, which 10 days previously had received 1.2 ml of fuadin solution, was sacrificed. A sample of the blood was defibrinated and centrifuged. The red cells were washed repeatedly with 0.9% NaCl, then suspended in 0.9% NaCl solution, and laked by shaking with a little ether. Cell stroma were removed from one portion of the mixture by centrifugation and from another portion by mixing with aluminum hydroxide and filtering. The hemoglobin was then isolated. The activities of samples, counted after dilution to 10 ml, are shown in Table II. Wintrobe(3) gives 13.0 ± 1.2 as the % of hemoglobin in rat blood. Even if the lower figure, 11.8%, is assumed, the activity of the hemoglobin fraction was sufficient to account for all the activity in the defibrinated blood.

One further step was to check the possibility that antimony might have taken the place of iron in the hemin portion of the hemoglobin molecules. The method of Fischer(4) was used to isolate hemin from the pooled blood of 10 rats, which had received intravenous injections of fuadin 8 days before sacrifice. The activity of the hemin was found to be only 299 counts per minute per gram when counted dry, whereas the acetic acid mother liquor retained 2335 counts per minute per ml when an aliquot portion was counted by the usual liquid counting method. If all the antimony were held in the hemoglobin just as iron is held, the activity of the hemin fraction should be many times as great as that of the mother liquor. The antimony might

TABLE II. Antimony Content of Samples from Blood of Rat.

Sample	c/min./g
Defibrinated blood	8600
Plasma	65
Hemoglobin (aluminum hydroxide method)	73800
Hemoglobin (centrifugation only)	74100

have been loosely attached to the hemin, but it seems more likely that it was attached to the protein portion of the hemoglobin. An added reason for believing this is the fact that the red cells of the dog do not seem to take up the antimony nearly so avidly as those of the rat. Presumably there is no difference between the hemin of the rat and of the dog, but the protein portions of the molecules are different. Support by analogy is found in the fact that arsenic, a member of the same family of elements as antimony, when administered as sodium arsenite, has been shown to be taken up by the red cells of the rat and to go with the globin and acid-acetone soluble heme fractions(5).

Summary. Labeled fuadin prepared from neutron irradiated antimony trioxide has been used in a study of the localization of antimony in the red cell fraction of rat blood. The results are consistent with the hypothesis that the antimony is attached to the globin portion of hemoglobin.

The assistance of Edgar Cress, Wallace Luke, and Lewis Warren in the animal experiments is acknowledged with appreciation.

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The Polysaccharides of *Candida albicans*.* (21103)

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The presence of serologically active polysaccharides in organisms belonging to the genus Candida has held the attention of many investigators interested in devising serologic tests for the diagnosis of Moniliasis, and demonstrating cross reactions between Candida and other closely related yeast-like organisms (1-3). The present report studies the homogeneity of a *Candida albicans* polysaccharide preparation by quantitative immunologic technics.

Materials and methods. A strain of *Candida albicans*‡ giving typical chlamydospore formation on corn meal agar, sugar fermentation reactions consistent with the species, and requisite pathogenicity in the rabbit was selected for use in this work. The organism was cultured on Sabouraud's agar in Roux flasks at 30°C for 3 days. Growth was washed off gently with acetate buffer, pH 6.03, and adjusted to approximately 50% concentration by volume. The suspension was autoclaved at 15 lb pressure for 10 minutes and then centrifuged to recover the supernatant. Addition of one volume of 95% alcohol precipitated the polysaccharide and protein constituents, which after solution in distilled water were treated by Sevag's method to free it from protein. The purified polysaccharide, after additional alcohol precipitations in the presence of 10% sodium acetate and 1% acetic acid, was finally dialyzed against distilled water and dried by lyophilization. All procedures were carried out at 0-4°C. Gray rabbits weighing approximately 2 kg were injected intravenously with a heavy suspension of heat-killed *Candida albicans* cells over a 3-week period. Animals were injected 3 consecutive days of each week with increasing amounts of antigen. High titered serum was obtained by bleeding the rabbits 6 days after the last injection. The quantitative test was used and the technics outlined in Kabat and Mayer(4) for treating antigen-antibody precipitates prior to nitrogen determinations were followed. One ml of a 1:1 dilution of antiserum was added to tubes containing 4 ml of antigen dilution prepared in saline. The total antibody nitrogen was estimated quantitatively by a modification of the biuret method suggested by Robinson and Hogden(5). A standard curve was drawn from spectrophotometric density readings at 540 mM of the biuret color developed from amounts of purified egg albumen ranging from 0.5 to 10 mg of protein. The biuret color developed by antigen-antibody precipitates was read on this curve and the antibody nitrogen estimated by multiplying the protein value by 0.16.

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‡ Kindly supplied by Dr. Leanor Haley, Yale University School of Medicine.

Results. The values for antibody nitrogen precipitated by added antigen appears consistent with a single antigen-antibody system throughout the range of antibody excess; however, the absence of a clear-cut equivalence zone as determined by supernate analysis, and the persistence of insoluble antigen-antibody complexes with a marked increase in total antibody precipitated in the region of antigen excess, strongly suggests the inhomogeneity of the polysaccharide preparation(6) (Fig. 1 Curve A). Attempts were made to fractionate the polysaccharide preparation into 2 components by precipitation with saturated lead acetate. On one occasion lead precipitation removed a yellow, gummy material, apparently nucleic acid, since U.V. absorption at 2600 Å of material remaining in the supernate was greatly reduced after treatment. Separation of the 2 components, however, was not successful.

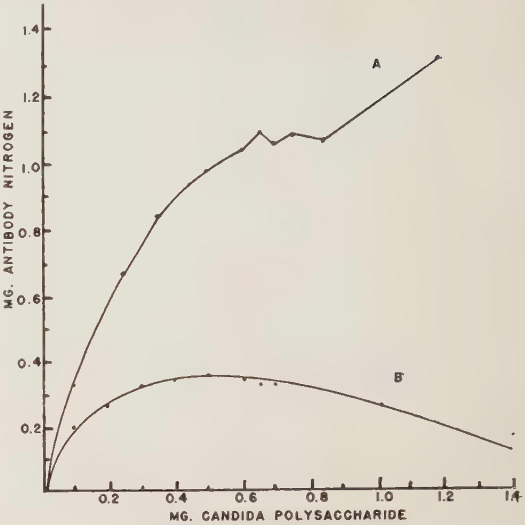

FIG. 1. Reaction of Candida polysaccharide in unabsorbed anticandida rabbit serum (Curve A) and anticandida rabbit serum absorbed with Saccharomyces polysaccharide (Curve B).

To confirm the presence of 2 distinct antibodies in the Candida antiserum, thus reflecting the impurity of the antigen preparation and allowing us to characterize the combining characteristics of the common and type-specific antibody, advantage was taken of the fact that *Saccharomyces cerevisiae* shares a common antigen with the Candida genus(7,8). Thorough absorption of Candida antiserum with Saccharomyces polysaccharide, prepared as described in methods, removed the greater portion of antibody while leaving behind a small fraction which still reacted with the Candida polysaccharide (Fig. 1, Curve B). Note that only after absorption of this common antibody was it possible to obtain a curve consistent with a single antigen-antibody system, showing the formation of soluble complexes and decreasing amounts of antibody precipitated in the region of antigen excess.

Similar experiments were conducted using Saccharomyces antiserum. Absorption of this antiserum with Candida antigen removed a major portion of the antibody and left behind a smaller fraction which still reacted with the Saccharomyces antigen. Thus it appears that rabbit antisera produced by either Candida or

Saccharomyces contain common antibody far in excess of the type-specific antibody.

Discussion. The separation of a type-specific antigen from common group antigen in polysaccharide preparations from *Candida albicans* would offer a distinct advantage when testing for humoral or tissue antibodies in the diagnosis of Moniliasis, since material used at present unavoidably detects antibodies to Saccharomyces as well as Candida. The preparation from *Candida albicans* appeared to contain 2 components, one rapidly soluble in distilled water and the other slowly soluble and gelatinous. While it has not been possible to separate 2 distinct components by chemical means, the rapidly soluble fraction would appear to contain the common antigen since the Saccharomyces polysaccharide preparation containing this common antigen is rapidly soluble in distilled water. Other methods for separating the components are being tried, *i.e.*, electrophoresis, chromotography.

The possibility that autoclaving the antigen accounted for its unusual activity can be discounted since the same pattern of activity was observed in experiments using polysaccharides prepared from saline washings of unheated Candida cells, and from aerated liquid medium cultures. Further evidence of inhomogeneity of the polysaccharide preparation was obtained using the gel diffusion method described by Ouchterlony(9), which has been found effective in characterizing antigens containing components of different specificity. When Candida antigen and Candida antiserum were allowed to diffuse toward one another through a semi-solid gel 2 distinct bands of precipitate were formed, whereas only one was seen when Saccharomyces antigen was substituted for the Candida antigen.

Summary. A polysaccharide preparation derived from autoclaved cells of *Candida albicans* or saline washings of unheated cells has been shown by quantitative immunogolic methods to contain a common antigen shared with *Saccharomyces cerevisiae* and a type-specific Candida polysaccharide antigen. Antiserum obtained from rabbits immunized with heat-killed *Candida albicans* or *Saccharomyces cerevisiae* contains considerably more antibody reacting to the common antigen than to the type-specific antigen.

The author wishes to acknowledge the kind assistance and direction given this research by Dr. H. P. Treffers, Professor of Microbiology, Yale University School of Medicine.

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Failure to Produce Obesity in the Rat Following Gold Thioglucose Injection.* (21104)

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Brecher, Waxler and co-workers(1-3) have shown that extreme obesity can be induced in one-third or more of C_3H and albino mice by an injection of gold thioglucose. These findings have been confirmed by others in albino mice(4), Swiss mice(5,6), and non-obese littermates of a strain of mice genetically-susceptible to obesity(6). The following data show that rats do not become obese after injections of gold thioglucose in dosages which are, respectively, less than, comparable to, or in excess of those which produce obesity in mice.

Materials and methods. Three groups of rats of Wistar stock, raised in our laboratory were used in this study: 1) 41 immature males, 2) 36 young adult males, and 3) 38 male and 18 female castrated animals. In the first group the experimental animals received a single intraperitoneal injection of gold thioglucose.[†] The remaining animals were untreated. In the second group half the number of animals which received each of the 2 doses of gold thioglucose were injected intramuscularly and half intraperitoneally. Ten animals were given amounts of sesame oil equivalent to those employed as the vehicle in the rats receiving the gold preparation. Half of this group were injected intraperitoneally and half intramuscularly. Six animals served as uninjected controls. The animals in the third group were castrated when they were 30 days old. The experimental animals were initially given a single intraperitoneal injection of gold thioglucose. Thirty-five days later 6 of the previously injected males were given a second dose and their weight followed for an additional 21 days.

Fifty-eight young adult male C_3H mice[‡] were employed to obtain data for comparison with the results obtained with the rats. The experimental mice received a single intraperitoneal injection of the gold preparation while the control animals were untreated. The treatment of the rats or mice in each group and the dose of gold thioglucose given to each group is shown in Table I. All animals were weighed at weekly intervals throughout the experimental period.

Results in rats. Group 1. No animal became obese (Table I) and at no time did the body weight of any experimental animal equal that of several of the control animals.

Group 2. Death occurred in all adult male rats which received 1 mg of gold thioglucose per g of body weight. Of those given 0.75 mg per g of body weight, 50% survived in both the intramuscularly- and intraperitoneally-injected groups. The experimental animals which survived treatment lost considerable weight initially, but regained their former status about 3 weeks later. By 60 days following the injection the average gain of the gold-injected animals was still 27 g less than in either control group. The data from intramuscularly and intraperitoneally injected animals are combined in Table I since the results were similar. The sesame oil vehicle exerted no detectable influence upon weight.

Group 3. In neither sex did the average body weight of the experimental animals surpass that of the control rats during a 10-week period following the injections. In none of the injected animals did the body weight exceed the range found in control rats. A second injection of gold thioglucose also failed to initiate the development of obesity. As might be anticipated from the results obtained in

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[†] Gold thioglucose, prepared as 50 or 100 mg/cc of sesame oil, was obtained through the courtesy of Dr. Edward Henderson of the Schering Corp., Bloomfield, N. J. Gold thioglucose is stated to be stable and to be 50% gold.

[‡] Obtained from Jackson Memorial Laboratory, Bar Harbor, Me.

TABLE I. Gain in Weight of Rats and Mice following Injection of Gold Thioglucose.

Group	Age (days) Body wt	Treatment	Dose gold thioglucose, mg/g body wt	No. of animals	No. surviving	Avg total gain in body wt	Duration (days)
Rats	18-26	G*	.5 to 1.6	32	12	230	60
(1)	30-60 g	0	—	9	9	254	
”	90	G	.75	12	6	51	60
(2)	225-386 g	G	1.0	8	0	—	
		Sesame 0.1	—	10	10	78	
		0	—	6	6	78	
”	80	G (1 inj.)	.6 to 1.4				70
(3)	140-310 g	♂		8	6	84	
		♀		7	4	72	
		G (2 inj.) ♂	.6 to 1.4 and .9 to 1.3	6	5	53	
		0	—				
		♂		24	24	110	
		♀		11	11	102	
Mice	50-60	G	.8	48			38
	13-27 g	Obese			10	11	
		Non-obese			9	2	
		0	—	10	10	2	

* G = Gold thioglucose.

these three groups of rats we have found in a subsequent experiment that rats which have been injected with gold thioglucose do not consume more food than uninjected control animals.

Results in mice. Table I shows the rate of growth in mice which became obese as compared with uninjected controls and with injected animals which did not develop obesity. The weight increased at a much quicker rate in obese than in control mice and the obese animals were still gaining weight rapidly at the termination of the experiment.

Discussion. The experimental procedures which did not produce overweight rats appear to be essentially similar to those which resulted in obesity in mice. The gold thioglucose was prepared in the same way by the same company. That the dosages were not too small in rats is indicated by the fact that only 41% of the immature animals in Group 1 survived, and that all of the rats in Group 2 which received 1 mg of the gold preparation per g of body weight died. This compares with 90% survival in the mice in the study of Waxler and Brecher(1) and 40% survival in our mice, after receipt of 0.8 mg per g of body weight. The period of observation far exceeded that necessary to detect the development of obesity in the mice. Mice given gold thioglucose exhibited accelerated gains in weight within 2 to 3 weeks in our study and in that of others(1-4).

Age, sex, and reproductive status are probably not factors in the difference between the results obtained in rats and mice. The span of age employed in these studies included those successful in studies of mice. Immature males and adult rats of both sexes were utilized; neither sex became obese in rats, but both did so in mice(1-4). Even castrated rats did not become obese as a result of the gold thioglucose, although castration increases the tendency to obesity in many species(7-10).

In the light of available information a genetic difference seems to be the most likely explanation for the failure of rats, and for the ability of mice, to develop obesity following injections of gold thioglucose. In this connection it should be noted that the white rat appears to have little tendency to develop obesity under ordinary circumstances(5), although a special strain which is prone to obesity has been reported(11).

Summary. White rats, originating from the Wistar strain, did not become obese following injections of gold thioglucose under experimental conditions that have resulted in obesity

in 4 strains of mice studied. It is possible that genetic factors influence the capacity to develop obesity after injection of gold thioglucose.

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Apparatus for Continuous Infusion of Microvolumes of Solution into Organs and Tissues.* (21105)

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An apparatus has been devised for continuous infusion of microvolumes of solutions over long periods of time directly into tissues. It has been used successfully to infuse chemical substances into small regenerating limbs of salamanders(1-4), but it could be adapted for use in fields of study other than that for which it was designed. In comparison to other devices for perfusion into blood vessels (5-7), the apparatus has the advantages of non-interrupted flow, of delivering volumes of the order of 0.001 cc or less per hour, and of simplicity in construction, operation and performance. It utilizes the principle of the hand-driven micrometer screws that are employed in microchemical analyses(8,9).

Three models demonstrate the essential mechanism which may be modified for specific needs. In each, the rotary movement of a clock motor (Telechron) is translated into the forward movement of a screw device which advances, directly or indirectly, the plunger of a small hypodermic syringe. The syringe in turn delivers solution into fine polyethylene tubing and thence into a glass capillary needle which is inserted into the tissue. In experiments on the salamander, clocks with a speed of one revolution in 4 hours are most useful. In the simplest model (Fig. 1) a mounted micrometer caliper, in which a screw clamp is substituted for the anvil to accommodate the syringe, serves as the advancing mechanism. The mechanism to couple the clock and micrometer consists of a metal tube attached to the shaft of the clock and slit along its length to engage the pins of an extension shaft connected to the barrel of the micrometer. The motor, revolving counter-clockwise, is supported by an angle plate mounted between 2 rails of the stand so that the clock and micrometer can be engaged or disengaged. A thumb screw passing through a slit in the plate locks the plate in a selected position. The coupling constitutes an automatic shut-off device since, with forward movement of the

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† I wish to acknowledge with gratitude the work of Mr. John Fitzgerald, supervising mechanician of the Department of Physics, Cornell University, in construction of the apparatus and introduction of his original ideas in redesigning later models; and to my former students, Dr. Ned Fader and Dr. Richard Sidman, for suggestions in elaborating and testing the early model.

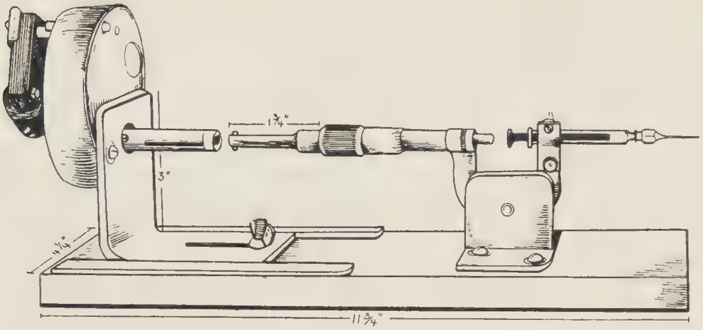

FIG. 1. Single-channel microinfusion apparatus.

caliper shaft, the coupling eventually disengages. The rate of forward movement is determined by the speed of rotation of the clock motor and the pitch of the caliper screw. The volume of solution delivered per hour may be determined by recording the forward movement of the plunger of the syringe over a long period of time.

A unit carrying a number of syringes is shown in Fig. 2. It incorporates the same principles of the simpler model except that a machined screw (stainless steel, ½ inch diameter, 40 threads per inch) is substituted for the micrometer caliper. The screw advances a horizontal plate attached to it by a bearing mechanism. The plate slides along parallel rods fastened to the upright members of the frame. One member carries the lead screw and the other is slotted to accommodate the syringes. The syringes are locked in place by swivel-mounted plastic plates. The advancing plate carries 4 adjustment screws arranged to contact the plungers of the syringes.

In the third model (Fig. 3) the screw (stainless steel, ½ inch diameter, 40 threads per inch—right hand) has no forward motion but is permanently mounted on ball-bearings to both frames. A sylphon bellows fixed to the end of the clock shaft engages the pins on the extension of the screw shaft by means of 2 slots. The coupling may be disengaged by compressing the bellows. The bellows permits immovable attachment of the clock support to the base but the advantage of the automatic release device of the previous coupling is lost. The advancing bar which bears the adjustment screws for contact with the plungers of the syringes carries an attached arm with clasp which rides firmly along a shaft between the 2 supporting members and insures that movement of the bar is in one plane. To preserve forward movement, a clockwise direction of rotation is required. The counter-clockwise direction of rotation of electric clock motors may be easily reversed by removing the coil from the armature, and

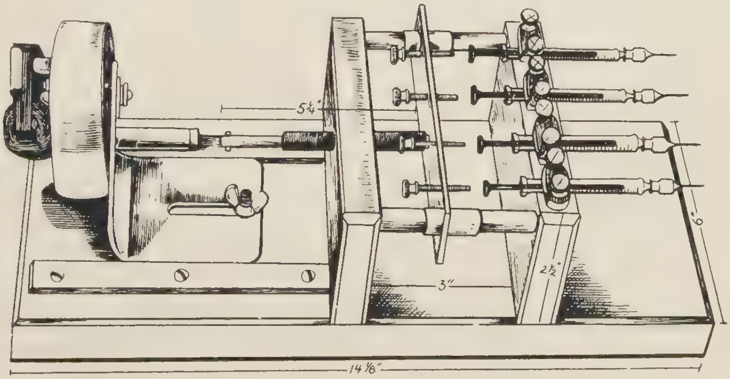

FIG. 2. Multiple-channel microinfusion apparatus with adjustable motor mount.

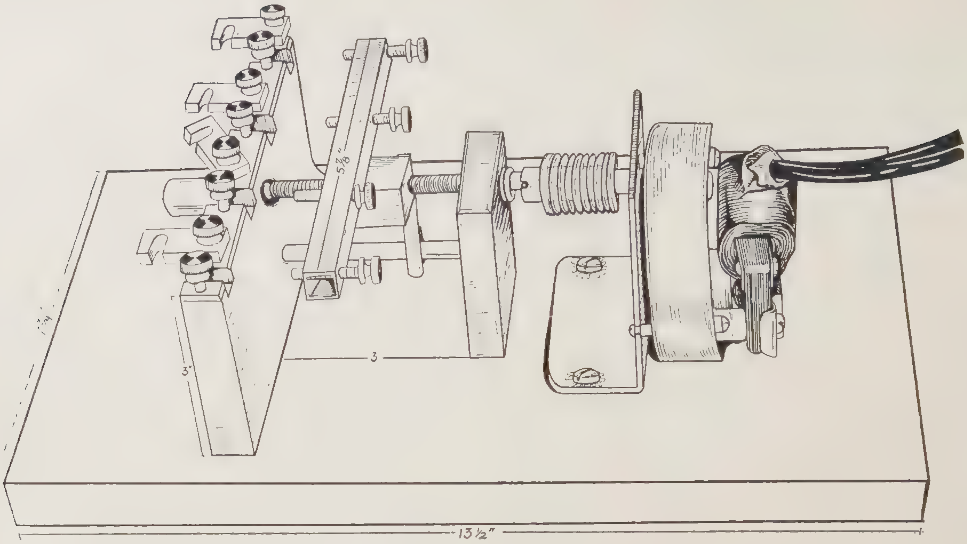

FIG. 3. Multiple-channel microinfusion apparatus with fixed motor mount.

replacing it in a reversed position.

There are a number of ways in which the mechanism can be further altered. Additional syringes can be driven by the same motor. The apparatus can be wired to feed discontinuously or to shut off following a prescribed time. By attaching the plungers of the syringes to the adjustment screws and reversing the motor it is possible to draw samples of tissue fluid into the syringes. A variable speed motor can be used instead of one with a fixed speed. Finally, syringes of any size may be employed.

Polyethylene tubing serves as leads from the needles of the hypodermic syringes. We have used ¼ cc tuberculin syringes, No. 26 syringe needles (½ inch) and tubing of inside diameter 0.015 inch. The tubing is sealed by heat to the needle and to the glass capillary tube at the other end, the glass having been previously drawn to a desired taper. Colored glass is of value in following the course of insertion in animal tissues. The plunger and mouth of the syringe are coated with silicone or fluorocarbon grease to avoid seepage.

Summary. An apparatus is described for infusion of small volumes of test solutions directly into tissues and organs. In more than 3000 successful infusions of the early regenerating limb of salamanders, volumes of 0.001 cc per hour have been introduced for periods of 5 or more hours. The apparatus consists of an advancing bar which is driven forward by a screw shaft attached to a clock motor. The advancing bar presses against the plungers of small syringes forcing fluid into fine polyethylene tubing and thence through a glass capillary needle into the tissues.

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Differences in Thermostability of Antigenically Related Strains of Poliomyelitis Virus.* (21106)

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Recent studies have shown that related strains of a virus may have different sensitivities to heat(1,2), and previous work from this laboratory has suggested that this might also be true for poliomyelitis strains(3). The earlier experiments have been extended in the present study which shows that 3 strains of poliomyelitis virus, all of the same immunologic type, differ in their susceptibility to heat. Because the differences are slight, a method (3,4) for their accentuation was used, *viz.*, the viruses were suspended in heavy cream or ice cream mix and heated in these media. A report on the pasteurization of milk and cream artificially contaminated with poliomyelitis virus has appeared elsewhere(3).

Materials and methods. Viruses. 1) *Lansing strain.* This poliomyelitis strain, received from Dr. Charles Armstrong, had been passed 275 times in mice and then 10 times in cotton rats. The latter material was used in these experiments. 2) *Y-SK strain.* This strain was isolated in this laboratory by Trask and Paul. After 21 passages in monkeys, it was adapted to cotton rats and mice. The strain used in these experiments had been passed 5 times in cotton rats. 3) *MEFl strain.* This strain was isolated by Dr. C. E. Van Rooyen and obtained from Drs. Jordi Casals and Peter K. Olitsky who adapted it to infant mice. The MEFl strain had been passed 86 times in infant mice and once in cotton rats, the latter being the source of virus for the heating experiments. *Preparation of virus suspensions.* For each strain the brains and cords of 20 infected cotton rats were thoroughly ground without abrasive until a homogenous mass resulted. To one portion, enough sterile distilled water was added to make a 10% suspension; to the other portions cream and ice cream mix were added in the place of water. The suspensions were centrifuged sufficiently to sediment tissue debris, but not at forces great enough to break the cream emulsion. *Suspending media.* The pasteurized heavy cream for these experiments was purchased on the open market. The ice cream mix (non-aerated) had been pasteurized before being used for the heating experiments. *Method of heating*(3). The virus suspensions were drawn into thin, U-shaped glass capillaries with an average outside diameter of 1.71 mm and with a length of 40 cm, and then both ends were sealed. These capillaries were tied with cotton thread, by means of which they were lowered until completely immersed into a water bath maintained at the desired temperature; immediately after heating for 15 or 25 seconds, the capillaries were dropped in ice water. The water bath employed was accurate to 0.05°C. *Determination of virus activity.* The rodent strains of poliomyelitis virus were inoculated intracerebrally in 0.03 ml amounts into 4-8-week-old Swiss mice and 4-6-week-old cotton rats. The animals were observed for a period of 4 weeks. Deaths which occurred within 2 days after inoculation were considered nonspecific. Passage was made of brain and spinal cord from those animals exhibiting typical signs as well as atypical ones, particularly those coming down following inoculation of material which had been heated at high temperature. A special point was made to pass material where only a small number in a test group developed disease or died without signs being observed.

Results. Effect of cream on the thermostability of virus. None of the 3 strains, Lansing, Y-SK, or MEF1 suspended in *water* withstood inactivation by heating at 71.1°C for 15 seconds. This was true regardless of whether the heated viruses were inoculated into cotton rats or mice (Table I). When the Lansing strain was suspended in *cream,* however, a small amount of virus resisted inactivation at this temperature. Increasing the temperature

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TABLE I. Effect of Cream on Thermal Stability of 3 Strains of Type 2 Poliomyelitis Virus.*

Strain of virus	Heated in water 71.1°	Heated in cream 71.1°	73.9°	76.7°	79.5°C	Original virus titer
		A. Tests in cotton rats				
Lansing	0/11	2/11†	0/12	0/11	0/12	$10^{-4.0}$
Y-SK	0/12	6/11	7/10	8/12	10/12	$10^{-3.8}$
MEF1	0/11	3/12	6/11	4/11	2/10	$10^{-2.8}$
		B. Tests in mice				
Lansing	0/36	9/48	2/46	2/29	2/43	$10^{-3.5}$
Y-SK	0/24	16/24	9/24	8/17	18/39	$10^{-2.5}$
MEF1	0/36	22/36	15/40	12/38	10/39	$10^{-3.0}$

* 10% spinal cords of infected cotton rats heated 15 sec. at indicated temperatures.

† 2/11 indicates that of 11 animals inoculated, 2 came down with disease.

to 73.9°C sufficed to inactivate the virus to the point where none of the inoculated cotton rats came down. As the results in Table I show, Y-SK proved to be more thermostable than did Lansing, and the virus persisted, despite an increase of the temperature to 79.5°. Ten cotton rats developed experimental poliomyelitis out of the 12 inoculated with Y-SK heated at this temperature. The difference between Lansing and MEFl was less marked, but the latter strain also remained active even at this high temperature, 2 out of 11 inoculated cotton rats being infected.

The results were less striking, but nevertheless comparable with those obtained with cotton rats, when mice were used as the test animals. Thus traces of Lansing virus suspended in cream persisted even when the suspension had been heated at 79.5°C for 15 seconds. However, of 43 mice inoculated with Lansing, only 2 showed signs of infection. In contrast to this, 18 of the 39 mice inoculated with Y-SK heated under the same conditions came down. The MEFl strain behaved intermediately between the other two.

Effect of ice cream mix on thermostability of virus. Lansing virus suspended in *ice cream* was heated for 25 seconds with the result that none of the cotton rats came down after an inoculation with virus held at 71.1°C. Despite the 10 second increase in the heating time, a similar increased resistance of Y-SK was observed. Again, as the results in Table II indicate, Y-SK virus persisted up to a temperature of 79.5°C with more than half (7 of 11) of the cotton rats inoculated coming down with disease. Raising the temperature to 82.2, however, sufficed to inactivate the virus to such an extent that it was no longer detectable in cotton rats. The MEFl strain showed a slightly greater resistance than Lansing to heat in that 2 of the 11 cotton rats inoculated with virus heated at 71.1°C were paralyzed, but at 73.9°C virus could no longer be detected.

The observation that mice were more susceptible to the 3 strains after heating in cream was made again with virus suspended in ice cream (see bottom half of Table II). Lansing virus suspended in ice cream remained viable even though the temperature was elevated to 82.2°C: 3 of 42 mice became paralyzed following inoculation. The Y-SK strain remained infective at this temperature and as in all the previous experiments, more of the animals (10 of 29) came down than in the case of the Lansing strain. The MEFl strain appeared to be as resistant as Y-SK until the temperature of 82.2°C was reached; it was inactivated at this point to about the same extent as Lansing virus.

Discussion. Although the 3 rodent-adapted strains of Type 2 poliomyelitis virus, Lansing, Y-SK, and MEFl, are immunologically indistinguishable, certain differences have been noted in the past to exist between at least the first 2 strains. Monkeys could be infected when given the Y-SK strain of virus orally, but they were not susceptible to infection with Lansing by this route of inoculation(5). In the growth of Lansing and Y-SK viruses in monkey testicular tissue culture(6), only the latter strain proved cytopathogenic even though both viruses multiplied to the same

TABLE II.
Effect of Ice Cream on Thermal Stability of 3 Strains of Type 2 Poliomyelitis Virus.*

Strain of virus	Heated in water 71.1°	Heated in ice cream 71.1°	73.9°	76.7°	79.5°	82.2°C	Original virus titer
			A. Tests in cotton rats				
Lansing	0/11	0/11	0/11	0/10	0/10	0/12	$10^{-4.0}$
Y-SK	0/12	5/12	8/12	6/12	7/11	0/12	$10^{-3.3}$
MEF1	0/11	2/11	0/11	0/10	0/11	0/10	$10^{-2.8}$
			B. Tests in mice				
Lansing	0/35	9/38	10/40	7/40	5/45	3/42	$10^{-3.5}$
Y-SK	0/24	12/21	8/22	6/19	12/27	10/29	$10^{-2.5}$
MEF1	0/37	18/42	13/31	16/41	15/41	2/36	$10^{-3.0}$

* 10% spinal cords of infected cotton rats heated 25 sec.

degree, according to titrations of infectious culture fluid in mice.‡ To these differences between the strains, one may now add the greater thermal resistance of the Y-SK virus. To detect this difference in response to heat by the 3 strains, it is apparently necessary to suspend them in a protective medium such as cream or ice cream mix(3), for in no case did any of the strains prepared in water suspension resist thermal inactivation at 71.1°. The cream probably slows the rate of inactivation sufficiently to magnify the differences between the strains. The differences in the thermostability between the 3 strains did not appear to be due to a greater concentration of one over the other in the suspensions being heated. This is emphasized by the fact that in every one of the experiments reported here the titer of Lansing was from 0.7 to 1.0 log higher than that of Y-SK.

The present results confirm and extend earlier observations on differences in the thermostability of these strains(3): Lansing strain suspended in milk was inactivated by heating at 50°C for 30 minutes, whereas Y-SK proved resistant to this treatment. When suspended in cream, and heated at 71.1°C, the Lansing strain was inactivated after holding time of 45 seconds, whereas the Y-SK strain remained infectious until it had been held at this temperature for 180 seconds.

As far as we are aware, this is the first reported instance of a difference in the response of 2 related poliomyelitis viruses to a physical agent like heat. The fact that 2 such closely related strains can differ in their thermostability is, however, not a new observation in the field of virology. Burnet and Lind(1) have found that the 3 virus types derived from the classical WS strain of influenza differ in their heat resistance, and they have used these differences as genetic markers. A similar phenomenon has been reported by Adams(2) for a heat resistant mutant of coliphage T5 which appears to be identical to the heat susceptible phage in all of its other properties. Thus, we now have a means for the characterization of Lansing and Y-SK strains of poliomyelitis virus which perhaps may be used as a marker in studies of viral genetics.

Previous work(3) has shown that when human fecal strains of poliomyelitis virus were suspended in milk or cream, they were destroyed by pasteurization. It is highly unlikely that dairy products in nature would ever be contaminated so heavily that they would contain the concentration of virus used in our experiments, but if so, and if the contaminating viruses were as stable as Y-SK, the present conditions of high-temperature short-time pasteurization would be inadequate for thermal inactivation.

Summary. Three rodent-adapted strains of poliomyelitis virus, all belonging to the same immunologic type, were found to differ in their thermostability; these were, in decreasing order of resistance, Y-SK, MEFl, and Lansing. The differences between these strains became evident only when they were suspended in cream or ice cream before being heated. All three strains suspended in water were inactivated by heating at 71.1°C for 15 seconds. When heated in cream the viruses withstood 79.5°C for 15 seconds and in ice cream they withstood 82.2°C for 25 seconds, with Y-SK resisting inactivation to the greatest extent.

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‡ Infected mouse cords were used as inoculum in these experiments(6). Phillips has shown that about 30 serial passages in tissue culture were required of this Lansing strain before it became cytopathogenic (7).

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Effects of Plastic and Steel Surfaces on Clotting Time of Human Blood.* (21107)

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Studies in our laboratories using a mechanical heart pump(1) and intravascular prosthetic devices(2) have stimulated an investigation of the effects of various plastic and steel surfaces on the clotting of human blood. Only a few relatively limited comparative studies of surface effects on clotting have been reported previously(3-6).

Materials and methods. A method for the determination of clotting time on various types of surfaces was devised in which all observations were made by one individual. Materials studied were identically shaped into biconcave receptacles or open-end curved tubes with radii of ½ inch and 2 inches. Prior to each measurement, the surfaces under study were thoroughly cleaned with an alkali detergent, rinsed in distilled water, and oven dried. A plastic box with a removable transparent cover was suspended in a constant (37 ± 1°C) temperature water bath and continually rocked by a slow speed motor through an arc of 70° at a rate of 15 cycles per minute. Within the box, 2 test surfaces were placed simultaneously. Blood was obtained from normal individuals with no clotting defects. For each pair of determinations, 10 cc of blood were drawn from an antecubital vein, through a sharp 18-gauge needle into a siliconed syringe. Blood was drawn immediately after placement of a tourniquet, and samples were discarded if the vein was not immediately entered. Two cc of blood were then placed on each of the 2 surfaces to be tested and the first 6 cc drawn into the syringe were discarded. Thus, the blood tested contained a low tissue thromboplastin concentration(7). Time was measured with a stop-watch. Initially, 2 times were recorded; the onset of clot formation, and the time of completion of the clot. It was found

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* The authors gratefully acknowledge the assistance of Mr. Philip Pfaff of the Instrument Shop of the National Bureau of Standards, who constructed the apparatus described and machined and polished the steel test surfaces and several of the plastic surfaces. We are indebted to the following companies for supplying us with their products: Armour Laboratories for A-15 Needle Coating Agent; the U. S. Stoneware Corp. for specially molded Tygon tubing (Tygoflex 75); the M. W. Kellog Co. for a machined and polished Kel-F receptacle; E. I. Dupont de Nemours and Co. for supplies of polyethylene (Alathon) and Nylon FM-3001; and Rohm and Haas and Co. for a large piece of methyl methacrylate (Plexiglas II). The surgical procedure described was directed and performed by Dr. Charles A. Hufnagel, Georgetown University School of Medicine.

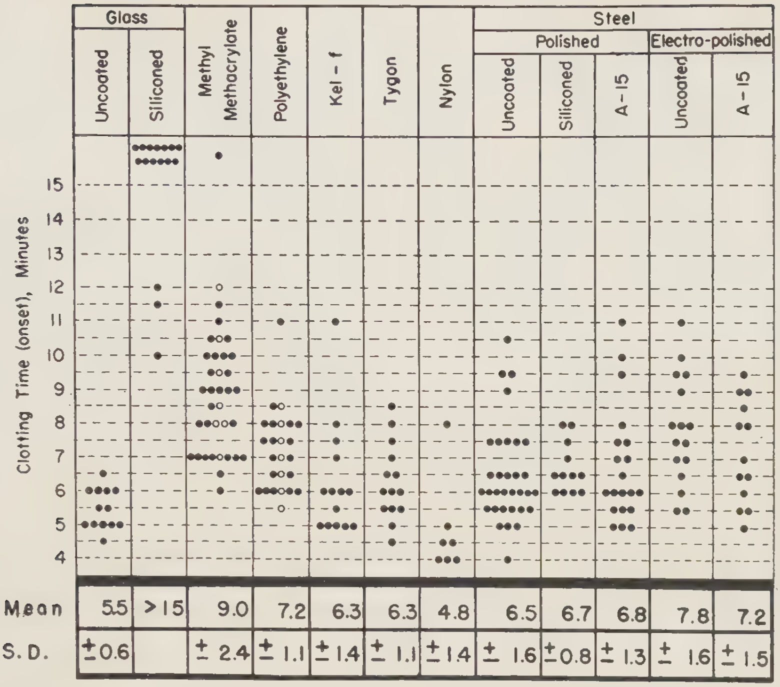

FIG. 1. Measured clotting times on the various surfaces, with means for each surface tested and standard deviations. Each point represents a single determination. Measurements made on surfaces soaked in water for 90 days are shown by the open circles and are not included in the calculations for mean and S. D.

that the onset of the clot had a more definite and reproducible endpoint, and this became the standard of measurement. The early clot was seen adhering to the surface of the test material in most instances, although in the cases of less "wettable" surfaces such as silicone, the earliest sign of a clot was a small dark floating fragment. If no clotting was noted in 15 minutes, the blood was poured on a tile surface and examined for evidence of clot formation. The materials studied are listed in Fig. 1. A-15 is a cationic nitrogen compound provided undiluted as follows: Tris (2-hydroxyethyl) dodecyl ammonium chloride, 5%; isopropyl alcohol, 40%; and distilled water, q.s. It was applied by boiling the alkali-cleaned steel test receptacle in a 10% solution for 15 minutes.

Results. The data are summarized in Fig. 1. Siliconed glass inhibited clot formation to a greater extent than any other material tested. Repeated determinations following one application of silicone revealed a rapid decline in this property. A second clotting time determination on a siliconed glass surface was not appreciably shorter than the first, but the third, fourth and fifth determinations revealed a rapid change in the surface effect. In these instances, the test surfaces were only saline rinsed between determinations. Fig. 1 includes only measurements made on the first application of silicone.

Methyl methacrylate exhibited a significantly greater clot inhibiting effect than other plastics, followed by polyethylene. Unsiliconed glass surfaces promoted clotting more readily than the polished No. 304 stainless steel surfaces. However, electropolished steel surfaces may have prolonged clotting times more than hand or machine polished steel surfaces. A-15 coated steel did not inhibit clotting further than uncoated steel. Clotting times on polished nylon (FM-3001) were shorter than those on glass or any other material tested.

Silicone applied to a steel surface did not significantly alter its effects upon clotting time, but the appearance of blood placed on this surface was similar to that of blood placed on a siliconed glass surface in that a "repellant" effect was observed. Although there was a demonstrable decrease in the "wettability" of the surface or the adhesive force between the blood and the surface, clotting time was not altered. As in the case of collodion-lined glass tubes(8), the rule that the clotting time is inversely proportional to the "wettability" of the surface(9) did not hold.

Plastic materials absorb definite proportions of water on soaking for variable periods, but a small number of determinations performed on methyl methacrylate and polyethylene surfaces soaked in water for 90 days revealed no significant differences in mean clotting times. When a high polish was obtained on plastic or steel surfaces, additional polishing did not further prolong clotting times.

Fig. 1 shows that the distribution of observed clotting times for any one material tested was not symmetrical. The general direction of the spread was toward prolongation of the clotting time. We note this phenomenon to have occurred in another study similar to ours(6). It implies that the method of study was adequately controlled for factors causing premature clotting, but not for factors which might inhibit some process in the normal clotting mechanism.

Walter, Murphy, Jessiman, and Ahara(6) studied the clotting time of blood on No. 302 stainless steel surfaces treated with A-15 and a group of related compounds using a technic similar to ours. In their investigation, a significant clot retarding effect was noted on coated surfaces. Differences in the methods of study and analysis of data do not seem sufficient to explain the marked differences in the conclusions reached by these authors and ourselves.

The demonstration that a highly polished, uncoated stainless steel surface does not promote clotting more than glass led to preliminary trials of intravascular prostheses fabricated of stainless steel. A 2½" length of stainless steel tube, ⅜" I.D., highly polished, was substituted for a short segment of aorta in a dog, by the "multiple point fixation" method of Hufnagel(2). Examination of the prostheses and adjacent aorta after 3 weeks revealed no evidence of clot formation.

Summary. A standardized method for the measurement of clotting time on surfaces of different materials has been described. Several glass, plastic, and stainless steel surfaces, and surface coating agents, were studied for their comparative effects on the clotting of whole human blood. The results may provide an indication, with regard to clotting, of the suitability of these materials for use in the construction of blood systems and vascular prostheses.

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Heat Stability Studies of Clotting Factors in "Thromboplastic" Preparations.

(21108)

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"Thromboplastin" in spite of being widely employed is not well characterized and defined. Generally, "thromboplastin" has come to embrace diverse tissue preparations which accentuate the formation of thrombin from prothrombin-containing systems, regardless of their mode of action. In practice "thromboplastic" activity is usually judged by the ability of a given preparation to accelerate the clotting of recalcified citrated or oxalated plasma. In addition, differences in nomenclature and definitions have caused a great deal of confusion with regard to the functional role of tissue preparations in the mechanism of blood coagulation. Following the nomenclature proposed by Milstone(1), thrombokinase is defined in this paper as an agent which has the capacity of directly converting prothrombin to thrombin.* The term thrombokinase was first introduced by Morawitz(2). Thromboplastin is defined in this paper as an accessory factor, which by itself cannot directly activate prothrombin but first reacts with another plasma factor, such as proconvertin, in the presence of calcium ions to yield a thrombokinase-like agent. The concept of a thromboplastic substance was first introduced by Nolf(3). The findings of Owren(4) made it evident that it is necessary to use thrombokinase and thromboplastin as distinct terms. Jensen, Gray, and Schaefer (5) reported that the clotting times of a $BaSO_4$-eluate from normal oxalated plasma with either platelets and $CaCl_2$ or with a commercial preparation of "thromboplastin," heated for 5 minutes at 70°C, are similar in both one-stage and 2-stage tests. These results indicate a similarity in the role of platelet factor and thromboplastin in the clotting mechanism.

In the present study the nature of the clotting factors present in brain extract was further investigated.

Materials and methods. Preparation of plasma. Rabbit blood was collected by cardiac puncture using the multiple syringe technic. The blood was drawn into an ice-cooled, silicone-coated syringe containing 1/10 volume of 0.1 M $K_2C_2O_4$ solution. Blood and oxalate were well mixed, delivered into ice-cooled, silicone-coated conical centrifuge tubes and centrifuged at 3000 rpm for 30 minutes at 0 to 5°C. Plasma was drawn off with a pipette, care being taken to minimize contamination of the plasma with platelets.

Preparation of platelets from rabbit blood. Blood was drawn by cardiac puncture as described above using 1/10 volume of 1% Di-sodium versenate, pH 7, as anticoagulant. Blood was delivered into ice-cooled, silicone-coated conical centrifuge tubes and centrifuged at 550 rpm for 25 minutes at 0 to 5°C to remove the erythrocytes. The platelet-rich supernatant plasma was drawn off with an ice-cooled, silicone-coated syringe. This plasma was centrifuged in ice-cooled, silicone-coated conical centrifuge tubes at 1700 rpm for 30 minutes at 0 to 5°C to pack the platelets. The supernatant plasma was decanted and the packed platelets were washed 2 times with volumes of 0.9% NaCl equal to the original plasma volume. After the second wash, the packed platelets were frozen. Before use, the platelets were thawed, suspended in 0.9% NaCl to the desired concentration and homogenized in a glass homogenizer. Commercial "thromboplastin"† was made up according to the manufacturer's directions. "Thromboplastin" was prepared from rabbit brain tissue according to the method of Quick(6). Resin (Rohm and Haas IR-105 (Na)) was employed to remove calcium ions. The essential details of representative individual experi-

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* According to Milstone(1) conversion of prothrombin to thrombin by thrombokinase can also take place in the absence of calcium ions.

† Simplastin, Chilcott Laboratories, Inc., Morris Plains, N. J.

TABLE I. Effect of Heat on Ability of Thromboplastin* and of Rabbit Brain Tissue† to Clot Oxalated Rabbit Plasma.

Test mixture‡	Clot formation times .066 mg $CaCl_2$	.56 mg $CaCl_2$
0.2 ml oxalated plasma	NC20′	5′37″
A. Thromboplastin susp. incubated 10 min. at 37°C, then filtered through resin:		
No treatment	30″	19″
Heated 5 min. at 56°C	50″	22″
20 60	NC20′	41″
5 70	1′11″	29″
B. Thromboplastin filtered through resin:		
No treatment	38″	23″
Heated 5 min. at 56°C	53″	26″
20 60	NC20′	48″
5 70	1′50″	33″
C. Brain tissue susp. in 0.9% NaCl incubated 10 min. at 37°C:		
No treatment	15″	18″
Heated 5 min. at 56°C	21″	24″
20 60	NC20′	1′26″
5 70	47″	45″

* Simplastin, Chilcott Laboratories, Morris Plains, N. J., made according to manufacturer's directions.

† 1 mg brain tissue/0.1 ml suspension.

‡ 0.1 ml thromboplastin-$CaCl_2$ or brain tissue-$CaCl_2$ mixture + 0.2 ml oxalated plasma. When 0.9% NaCl was substituted for $CaCl_2$, no clotting occurred.

ments are included in the various tables.

Results. The data presented in the various tables constitute average values of several identical experiments. The results presented in Table I indicate that heating of a "thromboplastin" preparation or of a rabbit brain tissue suspension at 60°C for 20 minutes destroyed the ability of both preparations to produce a clot when added to oxalated normal plasma in the presence of an amount of $CaCl_2$ not sufficient to produce any recalcification effect. Heating of these "thromboplastic" preparations at 56°C or 70°C for 5 minutes caused a partial destruction of this activity. In the presence of an amount of $CaCl_2$ sufficient for recalcification, clotting times were obtained which were much shorter than the plasma recalcification time.

The data in Table II show that the ability of brain extract to clot oxalated plasma in the presence of $CaCl_2$ not sufficient to produce any recalcification is lost after being incubated at 60°C for 10 minutes. In the presence of $CaCl_2$ sufficient for recalcification the activity also decreased somewhat on heating but the clotting times obtained were greatly reduced when compared with the plasma recalcification time. However, it is obvious that the clotting activity with regard to oxalated plasma in the presence of $CaCl_2$ not sufficient for recalcification is much more labile to heat than the clotting activity in the presence of $CaCl_2$ sufficient for recalcification. Heating of brain extract at 64°C for various lengths of time gave results similar to those obtained at 60°C. A comparison of the data of Tables II and III shows that a small amount of brain tissue will not clot oxalated plasma in the presence of an amount of $CaCl_2$ insufficient to produce any recalcification effect.

The data in Table III indicate that the heat (60°C) stability of thromboplastic activity of brain tissue and platelets is of about the same magnitude. The brain tissue and platelets were employed in comparable amounts with regard to their ability to accelerate the clotting of recalcified plasma. According to Soulier and Larrieu(7) 3 minutes at 64°C are sufficient to destroy completely the antihemophilic activity in a suspension of platelets, and 30 minutes destroys, in addition, a large part of a semithermolabile thromboplas-

TABLE II. Effect of Heat (60°C) on Rabbit Brain Tissue.*

Test mixture†	Min. incubation	Clot formation times .066 mg $CaCl_2$	.56 mg $CaCl_2$
0.2 ml oxalated plasma		NC20′	6′ 7″
0.1 ml brain tissue-$CaCl_2$ mixture + 0.2 ml oxalated plasma	0	12″	12″
	5	2′17″	21″
	10	NC20′	29″
	17	″	47″
	25	″	1′ 8″
	30	—	1′10″
	40	—	1′27″
	50	—	1′28″
	60	—	2′ 4″
	70	—	2′ 9″

* 1 mg brain tissue/0.1 ml suspension.

† Brain tissue-$CaCl_2$ mixtures incubated at 60°C. At time intervals shown, 0.1 ml mixture added to 0.2 ml oxalated plasma.

TABLE III. Effect of Heat (60°C) on Ability of Rabbit Brain Tissue* and of a Platelet Homogenate† to Clot Oxalated Rabbit Plasma.

Test mixture		Min. incubation at 60°C	Clot formation times .066 mg $CaCl_2$	Clot formation times .56 mg $CaCl_2$
.2 ml oxalated rabbit plasma + .1 ml saline	+ .2 ml $CaCl_2$	0	NC20′	8′31″
+ brain		0	"	1′50″
+ saline		10	—	9′40″
+ brain		10	—	6′ 1″
+ saline		20	—	10′13″
+ brain		20	—	7′21″
+ saline		30	—	9′36″
+ brain		30	—	7′13″
.2 ml oxalated rabbit plasma + .1 ml saline	+ .2 ml $CaCl_2$	0	NC20′	7′35″
+ platelets		0	"	2′19″
+ saline		10	—	8′ 7″
+ platelets		10	—	4′50″
+ saline		20	—	7′53″
+ platelets		20	—	5′13″

* .00625 mg/.1 ml suspension.
† 6× blood concentration.

tic lipoprotein, leaving a thermostable platelet factor.

Discussion. The effect of the "thromboplastin" preparations plus $CaCl_2$ (an amount not sufficient to produce any recalcification effect) on normal oxalated plasma is probably due to a direct activation of prothrombin, that is, to a typical thrombokinase action. In those cases in which an amount of $CaCl_2$ sufficient for recalcification is employed, tissue preparations react with another plasma factor forming a thrombokinase. When heated for 10 to 20 minutes at 60°C, the "thromboplastin" preparations apparently lost their thrombokinase activity since they failed to clot oxalated plasma in the presence of $CaCl_2$ not sufficient for recalcification. However, in the presence of $CaCl_2$ sufficient for recalcification, clotting times were obtained which were definitely shorter than the plasma recalcification times. The thrombokinase factor is obviously more labile to heat than the thromboplastic factor.

Quick, Hussey, and Epstein(8) found that the addition of rabbit brain extract to hemophilic blood will cause good consumption of prothrombin. When such an extract was heated to 60°C for 20 minutes, its ability to convert prothrombin to thrombin in hemophilic blood was lost, but it retained the ability to cause consumption of prothrombin in normal platelet-poor plasma. Biggs, Douglas, and MacFarlane(9) have expressed the view that human brain extract itself is not capable of converting prothrombin to thrombin but can form a thrombokinase-like substance upon reaction with factors V‡ and VII.‡

The data presented here and the observations of Quick and his associates(8) indicate that the indirect effect of thromboplastin must be distinguished from the direct action of thrombokinase on prothrombin in order to understand the action of brain tissue extracts. The clotting times obtained with tissue extracts may vary with their mode of preparation. The question of whether the presence of thrombokinase in brain extracts is derived from the blood trapped in the brain tissue, or is actually a constituent of the tissue, cannot be answered at present. The nature and degree of activity of tissue extracts may vary with the species of animal from which it is obtained.

The heat stable component of brain extracts apparently exerts a function in the clotting mechanism similar to that of the platelet factor. Both factors require the cooperation of another factor, such as proconvertin, before they are capable of converting prothrombin to thrombin. The similarity in action of platelet extract and brain extract

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‡ Factor V is the labile factor and factor VII of Koller(10) is proconvertin.

(heated to 60°C for 20 minutes) has also been pointed out by Quick and his associates(8). Whether the platelet factor and the thromboplastic factor of brain tissue are chemically identical has still to be determined.

Summary. 1. On the basis of heat stability studies, the clotting properties of brain tissue extracts are due to 2 components: a) a thrombokinase, which has the ability to convert prothrombin directly to thrombin, and b) a thromboplastin, which is an accessory factor and must react first with another plasma factor, such as proconvertin, before it is capable of converting prothrombin to thrombin. 2. The indirect effect of thromboplastin must be distinguished from the direct action of thrombokinase on prothrombin if the mode of action of so-called "thromboplastic" brain tissue extracts is to be understood.

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Levels of Serum Protein Fractions in Diabetic Patients with *Retinitis proliferans.** (21109)

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There has been much speculation as to the etiology of the degenerative vascular lesions seen in long-term diabetes. The question has been raised as to what role, if any, shifts in serum protein and, in particular, serum mucoproteins and lipoproteins, play in the development of such abnormalities(1-3). Changes in the major serum protein fractions in diabetes have been reported. Schneider, Lewis, and McCullagh(4) described changes in the albumin and β-globulin fractions of the serum from 31 diabetic patients with retinitis. The reduction in albumin and the elevation of the β-globulin fraction were more marked in the retinitis cases than similar changes observed in uncontrolled diabetics. That these changes may not represent the same thing was indicated by a difference in response to treatment. Rifkin and Petermann(5) have reported the results of an electrophoretic study of the serum proteins of patients with clinically established diabetic glomerulosclerosis. A decrease in the albumin and a significant elevation of the α_2-globulin were found in all 10 cases. The β-globulin was normal or only slightly elevated. Changes in the serum lipoproteins in diabetics with vascular degenerative changes have been reported by Keiding *et al.*(6) and by Engelberg *et al.*(7). The elevation of the S_f 12-20, S_f 21-35, and S_f 35-100 lipoproteins was found to correlate with the presence of both diabetic retinopathy and nephropathy. Changes in the serum mucopolysaccharides and mucoproteins in patients with vascular degenerative disease have been investigated systematically both by Berkman *et al.*(8) and by Keiding and Tuller(9). However, during the work of the latter authors it was evident that it would be of interest to follow changes in the major serum protein fractions along

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TABLE I. Clinical Characteristics of 14 Patients Studied.

Sex	Age, yr	Duration of diabetes, yr	*Retinitis proliferans*	Proteinuria	Hypertension	Renal insufficiency
♂	38	23	+	+	+	+
♂	34	18	+	+	+	0
♀	34	23	+	+	0	0
♂	37	26	+	+	+	0
♂	24	13	+	+	0	0
♂	24	2+	+	+	0	0
♂	33	20	+	+	+	0
♂	28	25	+	+	+	+
♀	28	19	+	0	0	0
♂	38	21	+	0	0	0
♂	58	21	+	0	0	0
♀	33	28	+	0	0	0
♀	70	14	+	0	+	0
♂	50	10	+	0	(+)	0

with the determination of the protein-bound carbohydrates in patients showing vascular disease in various stages. Two groups of diabetic patients were selected for study, one with marked retinal changes (*retinitis proliferans*) and no clinical evidence of kidney damage, and the other with both *retinitis proliferans* and renal damage.

The results of the determination of serum protein changes through the use of paper electrophoresis and of the analysis of protein-bound carbohydrates on the patients are reported below.

Methods. Blood was taken from patients before or 3 hours after breakfast. Analyses for protein-bound carbohydrates were carried out at once by 2 methods. The non-hexosamine carbohydrate content of an alcohol precipitate of the proteins in serum was determined by the anthrone method(10). Hexosamine content was determined by a modified Elson-Morgan technic(11). The concentrations of these protein-bound carbohydrates are reported as mg/100 ml of serum. *Paper electrophoresis* of the serum proteins was performed by the basic technic of Kunkel and Tiselius(12). Whatman No. 3MM filter paper was placed between siliconized glass plates after the application of 0.01 ml of serum to the paper. Separation was carried out for approximately 4 hours in a barbital buffer, 0.05 ionic strength, pH 8.6, and for applied voltage of 15 V/cm of paper. The albumin fraction moved approximately 12-13 cm. Bromophenol blue was used to stain the protein. The strips were evaluated by eluting 0.5 cm segments in 0.01 N NaOH and the color intensity read with a Klett-Summerson colorimeter. The readings were plotted and the areas under the curves determined with a planimeter. The concentration of each fraction is expressed in g/100 ml of serum. A strictly standardized technic was used, and normal serum was always run simultaneously with pathological serum to maintain maximum reproducibility and comparability. The average variation of separate runs of the same serum was: albumin plus α_1-globulin ±1%, α_2-globulin ±2.9%, β-globulin ±2.4%, and γ-globulin ±1.5%.[‡] Proportionality was found between protein concentration and stain uptake for the albumin fraction within the concentration limits used. Although the globulins did not take up the dye quite in proportion to protein concentration at high levels, the differences were small. Since comparison was made between normal and pathological sera run by the same technic, no correction factor was introduced. Determinations of the *total protein* were made using the biuret reaction as modified by Mehl(13) in 1945. Purified human albumin was used as the standard reference.

Material. Non-diabetic subjects used were doctors and laboratory personnel in the hospital. They were healthy, showed no signs of infection and no albumin in their urine. The group consisted of 4 females (ages 21, 38, 41,

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[‡] These determinations were checked in the laboratory of the Steno Memorial Hospital, Gentofte, Denmark.

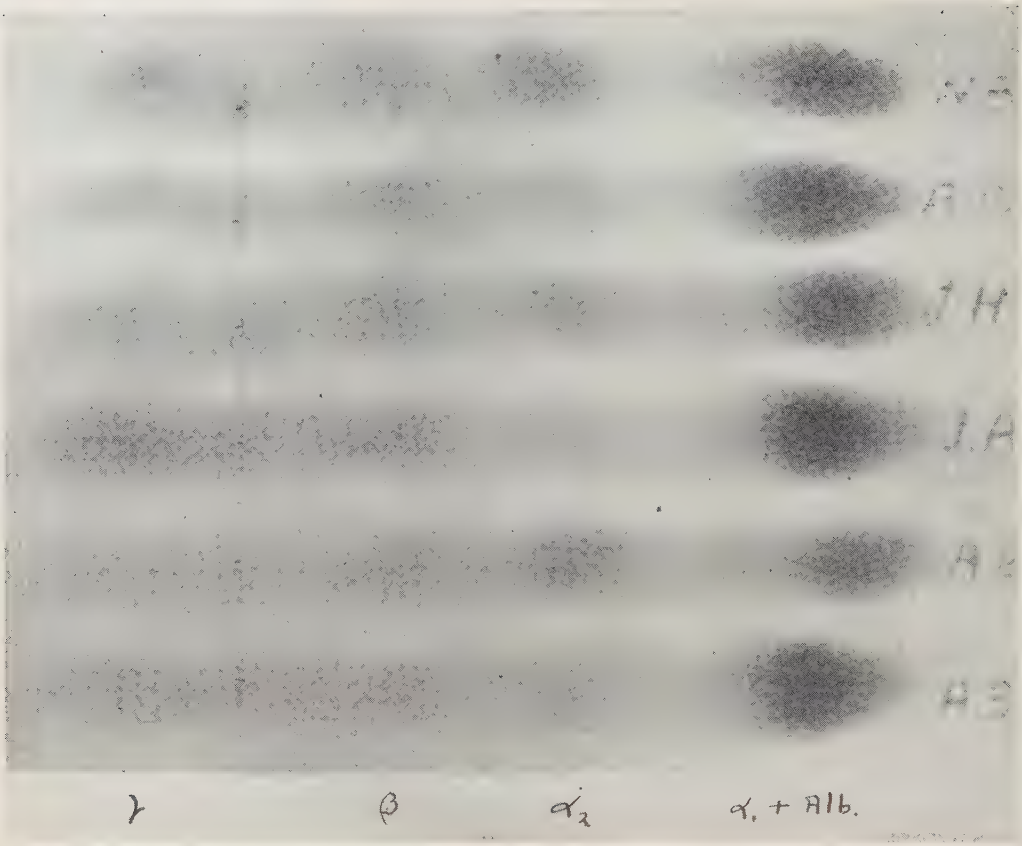

FIG. 1. Paper electrophoresis patterns of the proteins in representative sera. (B.C.), (J.A.) normal subjects; (N.B.), (J.H.), (A.G.) diabetics with *retinitis proliferans* and proteinuria; (A.S.) diabetic with early *retinitis proliferans* without proteinuria.

and 50 years) and 6 males (ages 22, 28, 32, 34, 37, and 37 years). The diabetics were patients in the New England Deaconess Hospital. The clinical data are presented in Table I. The diagnosis of *retinitis proliferans* was made in all cases by an ophthalmologist. Proteinuria was based on repeated findings of more than 20 mg % albumin in the urine. Azotemia was said to be present if the non-protein nitrogen concentration was elevated consistently above 40 mg %.

Results. The separation of serum proteins obtained in the paper electrophoresis is shown in Fig. 1. Six different sera are represented; 2 from normal subjects, 3 from diabetics with *retinitis proliferans* with proteinuria, and one from a diabetic with early *retinitis proliferans* without proteinuria. It is evident that there is a distinct increase in the α_2-globulin fraction in the diabetics with both *retinitis proliferans* and proteinuria, while there is only a slight increase in that of the diabetic without proteinuria. In the 3 diabetics with proteinuria, the serum albumin spot is less dense than in the non-diabetics.

Fig. 2 and 3 present graphically the quantitative changes in protein-bound carbohydrates and in the serum proteins respectively for the non-diabetics and the 2 groups of diabetics. As may be seen readily from Fig. 2, both groups of diabetics showed an increase in the protein-bound carbohydrate of both the non-hexosamine and hexosamine type. However, only in the group with proteinuria was there a change in total protein (Fig. 3).

From Fig. 3 it is evident that both groups of diabetics have a low combined albumin + α_1-globulin value in contrast to the shift in total protein. Likewise, both groups of diabetics show an elevated α_2-globulin value. The

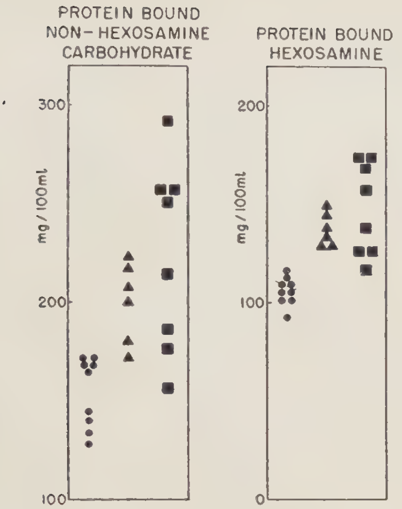

FIG. 2. Levels of protein-bound carbohydrate. ● Non-diabetics. ▲ Diabetics with *retinitis proliferans* without kidney disease. ■ Diabetics with *retinitis proliferans* and kidney disease.

β-globulin and γ-globulin values are not markedly different in the diabetics and non-diabetics. However, there does appear to be a trend toward higher levels of γ-globulin in the serum of diabetics with *retinitis proliferans* without proteinuria compared to the diabetics with both conditions.

Discussion. These findings of a disturbed pattern of serum proteins in diabetic patients with *retinitis proliferans* are comparable to other reports of protein disturbances in this disease within the limitations of differences in the quantitative methods. Thus, the discrepancy in the high β-globulin values reported in diabetic retinitis by Schneider *et al.* (4) and the almost normal values given here might be accounted for in this manner. The measurement in the Tiselius electrophoresis depends on the refractive index which changes with the quality and quantity of the lipoproteins while the paper electrophoresis measurements depend only on the amount of protein present and do not change with lipid content. That an increased concentration of lipoprotein in the β-globulin fraction is present in diabetes has been demonstrated by Russ *et al.* (14).

The decrease in the concentration of the combined albumin + α_1-globulin fraction and the increase in the α_2-globulin fraction is parallel to that reported by Rifkin and Petermann (5) in a study of 10 diabetics between 60-70 years showing "diabetic glomerulosclerosis," *i.e.*, all having marked proteinuria, hypertension, and edema.

In the data presented here, only 2 patients had renal disease advanced enough to show an elevated non-protein nitrogen. These and 3 others with proteinuria also had hypertension, and 3 had proteinuria as the only evidence of renal disease. However, a close rela-

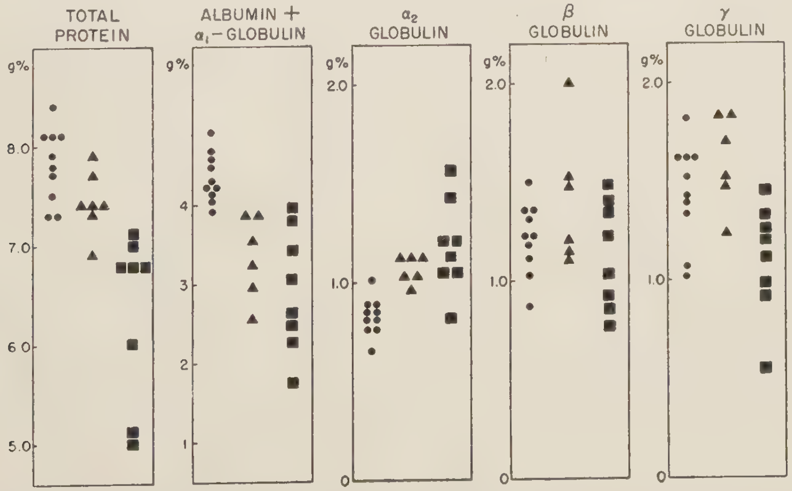

FIG. 3. Levels of total protein and of protein fractions in sera. ● Non-diabetics. ▲ Diabetics with *retinitis proliferans* without kidney disease. ■ Diabetics with *retinitis proliferans* and kidney disease.

tionship exists between *retinitis proliferans* and the Kimmelstiel-Wilson lesions as has been discussed by Root(2). Thus, it might be concluded from the data reported herein that the alterations in the serum protein pattern involving the albumin and α_2-globulin fractions are present in diabetic patients having *retinitis proliferans* and at a stage at which the protein changes cannot be accounted for by clinically detectable kidney damage. The protein changes become more marked with the development of clinical signs of kidney involvement.

The increase in concentration of total protein-bound carbohydrates found in this study roughly paralleled the increase in the α_2-globulin fraction. Similar parallelism was found by Seibert *et al.*(15) in their work on patients with tuberculosis. These authors on the basis of data obtained on Cohn fractions estimate the amount of carbohydrate bound to the α_2-globulin fraction to be 5.8%, whereas the amounts bound to albumin, α_1, β, and γ fractions are 0.1, 1.6, 2.7, and 1.6, respectively. Therefore, the variations of total protein-bound carbohydrates in the data presented here are probably principally influenced by the increase in concentration of the α_2-globulin fraction. Considerable influence on the total protein-bound carbohydrate value can be caused by variation in concentration of the β and γ fractions, but normally the content of bound carbohydrate is not so great in these fractions.

Summary. 1. The serum protein-bound carbohydrates and the serum protein fractions were studied in diabetic patients with *retinitis proliferans.* The serum protein-bound carbohydrates were found to be present in a higher concentration than in non-diabetes. By the method of paper electrophoresis, a definite decrease in the values for the albumin + α_1-globulin fraction and an increase in the α_2-globulin fraction was also found in all of the diabetics, although only those with both *retinitis proliferans* and proteinuria showed a decrease in the total protein. In the β-globulins no essential change was seen. In the group showing both retinitis proliferans and nephropathy, the γ-globulin concentration tended to be lower than in the group with *retinitis proliferans* alone, but none of the groups had values distinctly different from those of non-diabetics. 2. It is concluded that disturbances in the protein pattern exist which particularly involve the albumin and α_2-globulin fractions at a stage in the development of the diabetic vascular lesions where the changes cannot be accounted for by loss of protein in the urine.

The author is indebted to Miss Anna Gotsis for technical assistance and to Dr. Elizabeth F. Tuller for her help and cooperation in all aspects of the work and in the preparation of the manuscript.

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Ability of Rat to Adapt to Stress of Light Restraint.* (21110)

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(Introduced by U. D. Register.)
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In previous reports the phenomenon of thermoinstability induced by light restraint in the cold (*i.e.*, restraint hypothermia) has been observed(1,2). It has also been demonstrated that the emotionality of the animals is correlated with their ability to maintain body temperature while restrained in the cold, *i.e.*, the more emotional the animal the greater the drop in body temperature(3-5). Since animals are emotionally stressed by light restraint, it was wondered if they could become adapted to this stress. The present communication reports a study of the ability of the rat to adapt to the stress of restraint.

Methods and materials. To determine if animals could become adapted to the stress of light restraint, 30 young adult male Sprague-Dawley rats were exposed in restraining cages in the cold room for periods up to 7 hours and changes in the body temperatures were noted with indwelling rectal thermometers. They were then removed from the cold room and maintained in the same restraining cages for a week. During this period of "adaptation," food and water were given *ad libitum* as illustrated in Fig. 1. At the termination of the period of adaptation the animals were again exposed to the cold, while still in the restraining cages, and the changes in body temperature again noted in exposures of 7 hours. On removal from the cold room, the animals were taken from the restraining cages and maintained as were the other animals in the laboratory. At the close of this week in regular laboratory conditions, the animals were again placed in the restraining cages in the cold room and the changes in body temperature once again noted during a 6-hour exposure.

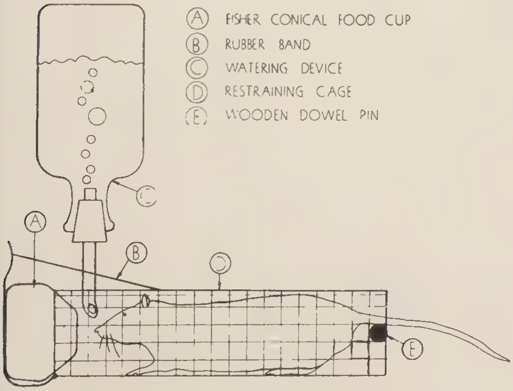

FIG. 1. Method used for giving food and water *ad libitum* during adaptation to stress of light restraint.

Results. In Fig. 2 is shown the body temperature drop of restrained conscious animals before adaptation (A), as well as the body temperature drop of the same animals similarly exposed to cold at the end of the adaptation period(B), and of the same animals one week after the adaptation period (C).

The responses of the animals in each group were arbitrarily placed into one of 3 categories: 1) animals which maintained an essentially normal body temperature and animals whose body temperature fell slightly and then rose to normal or near normal levels; 2) animals whose body temperatures fell gradually during the course of exposure but not falling below 25°C; 3) animals whose body temperature fell rapidly and progressively during the course of the exposure. The percentage of animals falling into each category is noted in Fig. 2.

Discussion. Since emotionality is involved in the phenomenon of restraint hypothermia (3-5), it seems probably that with suitable treatment the animals could become accustomed to the stress of light restraint so that they would remain relatively thermostable when so exposed in the cold. Since intermittent exposure to the stress of confinement results in a measurable increase in the resistance of the animals to the stress in the cold(1), it was thought that continual exposure to the stress of restraint might effect a greater degree

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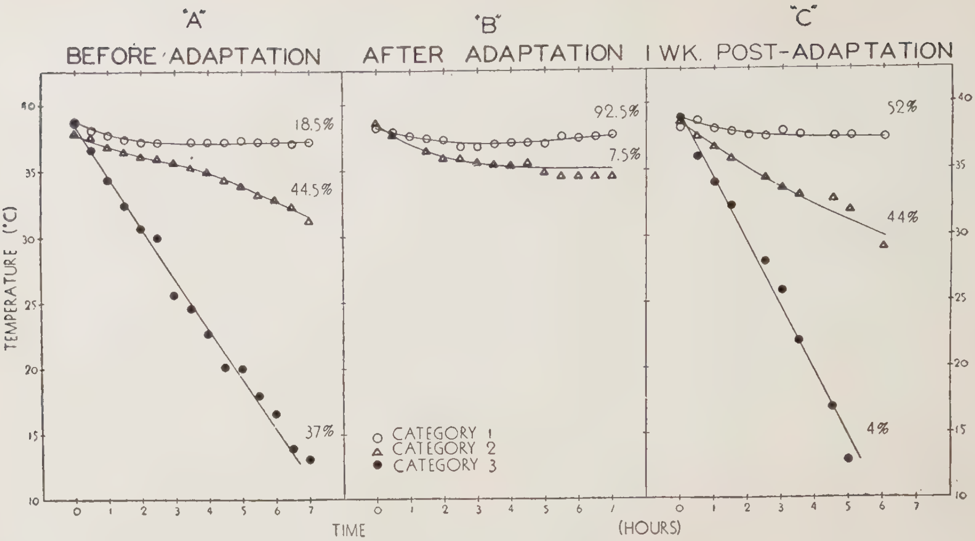

FIG. 2. Body temperature drop of rats exposed to cold. A. Before "adaptation." B. After "adaptation." C. 1 wk after "adaptation."

of thermostability in the animals. Thus, in this experiment, the restraining cages were fitted with food and water containers as shown in Fig. 1, and the animals exposed in them for a period of one week. During this time the animals became somewhat undernourished, losing from 10 to 50 g of weight. In spite of this condition of apparently lowered vitality, their response to caging in the cold, as shown in Fig. 2, showed a remarkable degree of thermostability as compared to their response before the period of adaptation. It has been determined(3) that restricted breathing is not a measurable factor in the hypothermia produced by light restraint. Since these animals were lightly restrained both before and after the period of adaptation, it is most probable that the improved response cannot be attributed to the greater breathing freedom afforded the animals after losing weight.

That some relatively stable change had been effected in the animals during adaptation is evidenced by the fact that the thermostability of these animals had not returned to the preadaptation level by one week after the completion of adaptation. Since the phenomenon of restraint hypothermia is possibly "triggered," as it were, by an emotional response, it is possible that this adaptation consists primarily in a "mental acclimatization" to the effects of the stress. However, even if the response of an animal to restraint in the cold is initiated by emotional factors, it is still possible that adaptation might involve increased adrenal cortical activity. In preliminary experiments in this laboratory, it has been demonstrated that cortisone injections impart a degree of resistance to hypothermia under the conditions of restraint in the cold. Biochemical assays of adrenal cortical activity are in progress which should further clarify this point.

Summary. Ability of the albino rat to adapt to stress of light restraint was investigated. Rats were able to adapt to the stress of restraint (for one week) in a cylinder of wire mesh so that they did not become hypothermic when exposed to the stress in the cold. The possible roles of emotional adaptation and adrenal cortical activity in producing the increased resistance to these stresses are discussed.

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Further Studies on Effect of Cold and Restraint on Tissue Non-Protein Sulfhydryl Compounds.* (21111)

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Recently Beck and Linkenheimer(1) reported a drop in concentration of liver non-protein sulfhydryl in unrestrained mice exposed to cold. This effect was not attributed to the observed lowering of body temperature (2). In a previous report(3) the effects of simultaneous exposure to cold and restraint on the liver and blood ergothioneine (ESH), glutathione (GSH), and total non-protein sulfhydryl (NPSH) concentrations were noted. Since a dual stress was used, the question of whether the marked decrease in liver GSH and NPSH was due to the low body temperature or to the stress of restraint was not answered. The present communication reports a series of studies in which attempts were made to quantitate the effect of hypothermia and the effect of restraint without a subsequent fall in body temperature on the concentration of tissue NPSH. Therefore, in an attempt to assay the separate effects, attempts were made to produce hypothermia in some animals without restraint and restraint in other animals without resulting hypothermia. Another group of animals was subjected to the dual stress of restraint and a drop in body temperature. The degree of alteration of the sulfhydryl compounds in these three groups was compared, in the present studies, with control values.

Methods and materials. Of the 122 young adult Sprague-Dawley rats used in the studies, 77 were females and 45 were males. The females were divided into 3 experiments, while all of the males were used in one experimental run. In each experimental series, the animals were divided into 4 groups. All treated animals were exposed for a period of 4 hours. One group consisted of untreated or control animals; a second group was restrained with body temperature maintained at normal levels; in a third group, animals were restrained and subjected to a cold environment. The rate of fall of the body temperature of this last group of animals was controlled at 5°C per hour so that the terminal temperature of the animals averaged approximately 19°C. A fourth group of animals was exposed unrestrained in the cold. Some of these animals were exposed in the cold room at a temperature of 0°C ± 2°C, while others were exposed in a deep freeze (temperature –15°C to –20°C). In the animals maintained unrestrained in the cold, no attempt was made to regulate the rate of fall of body temperature. The average terminal body temperature of each group is indicated in Table I. The animals were sacrificed with a blow on the head, the tissues excised immediately and frozen in powdered dry ice. Since ESH is not significantly altered by these treatments and since GSH comprises nearly all of the NPSH(3), the method of Benesch and Benesch(4) which measures NPSH was used. The changes which were observed, therefore, probably reflect changes in GSH.

Results. In Table I are shown the results of the experiments. It can be seen that both restraint and hypothermia were effective in lowering the sulfhydryl content of the liver. It is further noted that these effects are approximately additive (when the environmental temperature of the cooled unrestrained and the cold restrained animals were 0°C ± 2°); *i.e.*, neither restraint alone nor hypothermia alone produced the same decrease in concentration of NPSH as that produced by simultaneous exposure to hypothermia and restraint.

The cooled unrestrained animals maintained at the lower temperature (–15°C to –20°C) experienced a larger drop in concentration of NPSH as compared to the cooled unrestrained

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TABLE I. Effect of Cold and Restraint, Singly and Combined, on Liver Non-protein Sulfhydryl Compounds (NPSH).

Exp. No.	Treatment	No. of animals	Terminal body temp. (°C)	NPSH in μM %	t values
1 ♀	0 (controls)	7	38-40	786 ± 10*	
	Cooled unrestrained 0°C	7	36-39	699 ± 10	6.0
	Warm restrained	7	38-40	689 ± 47	2.0
	Cooled " 0°C	7	15-20	468 ± 24	12.0
2 ♀	0 (controls)	6	38-40	815 ± 23	
	Cooled unrestrained 0°C	7	36-39	740 ± 10	3.0
	Warm restrained	6	38-40	650 ± 22	5.1
	Cooled " 0°C	6	15-20	480 ± 29	9.1
3 ♂	0 (controls)	10	38-40	811 ± 21	
	Cooled unrestrained −15 to −20°C	16	15-35	552 ± 31	6.9
	Warm restrained	10	38-40	737 ± 23	2.4
	Cooled "	9	15-20	636 ± 45	3.5
4 ♀	0 (controls)	7	38-40	916 ± 11	
	Cooled unrestrained −15 to −20°C	6	15-35	553 ± 23	14.1
	Warm restrained	7	38-40	790 ± 32	3.3
	Cooled "	7	15-20	538 ± 23	14.7

* Stand. error of mean.

animals which were maintained at the higher temperature of 0°C. As indicated by the data, considerable hypothermia was produced in many animals by the lower temperature while little hypothermia resulted from a 4-hour exposure at the higher temperature.

No significant change in NPSH was observed in muscle or kidney tissue. The NPSH for muscle was 66-77 μM % and that for kidney was 350-450 μM %.

Discussion. From the data, it can be seen that both a drop in body temperature and restraint without a resultant drop in body temperature are effective in altering the sulfhydryl content of the liver. It is also apparent that under appropriate conditions these effects may be additive. As indicated by a recent publication from this laboratory(5), the diminution in concentration of these compounds may be a general response to stress. In this connection it is interesting to note, from the results in Table I, that the unrestrained animals which were maintained in the deep freeze experienced a greater drop in concentration of NPSH than did the animals which were maintained at 0°C. Although a greater degree of hypothermia was produced in those animals maintained at the colder temperature, individual cases indicated that a considerable decrease could be effected in concentration of NPSH without a significant fall in body temperature. In light, then, of a recent demonstration(5) of the effects of adrenalin on the concentration and distribution of these compounds, the results which were obtained in these experiments may simply be the results that would be obtained with the application of any general or specific physiological stress. If activation of the sympathoadrenal mechanisms is the more immediate cause of the alteration in the concentration of these compounds, it might be expected that the application of a dual stress (*i.e.,* hypothermia and restraint) would produce a greater alteration in the concentration of these compounds than would the application of either one of the stresses independently. Such relationships were obtained in the present studies. This increased effect would, of course, be referable to an increased stimulation and, therefore, an increased activity of the sympathoadrenal mechanism. The possibility of the existence of such a mechanism is further indicated by the fact that the unrestrained animals exposed in the deep freeze experienced a much greater drop in liver NPSH than did animals exposed at 0°C, which could well indicate increased sympathoadrenal response to the increased stress.

Several communications(6-8) have suggested the importance of these sulfhydryl compounds in the intermediary metabolism of carbohydrates and fats; therefore, any lowering of sulfhydryl compounds in the liver or other tissues of the animal might well interfere with these phases of metabolism. Therefore, in a stressful situation it would be advantageous for an organism to maintain adequate levels of these sulfhydryl compounds in organs and tissues where activity is necessary. An example of such organs or tissues is the muscle where no change in the concentration of these compounds was observed. Barron(8) suggested that GSH may function in maintaining enzymes in their active sulfhydryl form. In a stressful situation where metabolism is increased, it is possible that this activity of GSH is also increased. Therefore, some of the changes observed in this study may be due to the conversion of reduced glutathione (GSH) to oxidized glutathione (GSSG) which was not measured by the method used.

Summary. Both hypothermia and restraint were independently capable of decreasing the concentration of liver NPSH. When these stresses were applied simultaneously, the effect of the two stresses was approximately additive. No significant change of NPSH was observed in the muscle and kidney. The possibility that the more immediate cause of the drop in concentration of liver NPSH was activation of the sympathoadrenal mechanism is discussed.

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Influence of Testosterone Propionate on Protein Metabolism of Thyroidectomized Rats. (21112)

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Administration of testosterone causes weight gain and a decrease of the urinary nitrogen excretion of the normal(1), castrated (2), adrenalectomized(3), and hypophysectomized(4,5) rat. The protein anabolic effects of testosterone thus occur in the absence of the hormones of the gonads, of the adrenal glands, and of the anterior pituitary gland. The protein anabolic effect of testosterone differs from that of thyroxine in as much as the latter in physiological doses, causes weight gain and nitrogen retention in the thyroidectomized rat(6), but is ineffective in the normal(6) or the hypophysectomized rat(6). In view of the fact that testosterone treatment fails to restore the serous granules of the submaxillary gland of the thyroidectomized rat (7) and fails to cause increase of the liver protein in the thiouracil-treated rat(8), but does cause weight gain and urinary nitrogen retention in the thiouracil-treated rat(9), it was considered of interest to determine whether testosterone propionate would exert an anabolic effect in the thyroidectomized rat.

Methods. Ten male rats, descendants of the Wistar strain, weighing between 257 and 292 g were used. The animals were thyroidectomized 2 months prior to the experiment. All animals were tube fed the "mixed" diet described by Ingle *et al.*(10) twice daily. The animals were gradually adapted to tube feeding, and then were fed a constant amount during the control and the experimental periods. The animals were kept in wire screened, individual metabolism cages in a constant temperature room. The 24-hour

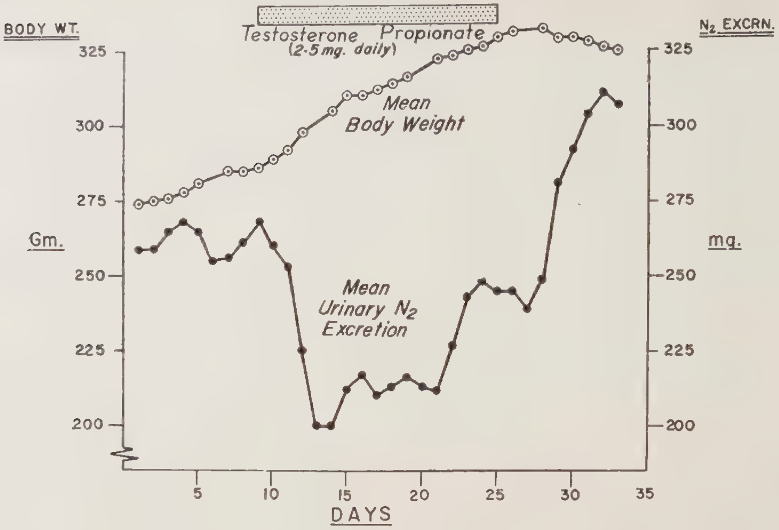

FIG. 1. Influence of testosterone propionate on body weight and urinary nitrogen excretion of thyroidectomized rats (mean of 10 animals).

urine specimens were analyzed daily by a micro-Kjeldahl method. The urinary nitrogen excretion and the weight of each animal were determined for a 9-day control period followed by a 16-day experimental period during which each animal received a daily dose of 2.5 mg of testosterone propionate by subcutaneous injection. The observations were continued for 8 days after the injections of testosterone propionate were stopped. After the completion of the experiment each animal was carefully inspected for completeness of the thyroidectomy.

Results. The results are recorded in Fig. 1. Testosterone caused a significant decrease in the urinary nitrogen excretion, and induced increased weight gain in the thyroidectomized rats. These changes are not evident until the third day of treatment. The urinary nitrogen excretion begins to increase on the 13th day of treatment, and this increase is maintained during continued treatment ("escape"). Following the cessation of treatment the urinary nitrogen excretion increases further, to levels higher than those of the control period; this "rebound" is associated with weight loss.

Discussion. Testosterone propionate induces weight gain and nitrogen retention in the thyroidectomized rat fed a constant amount of an adequate diet. The response of the thyroidectomized rat is similar to that of the normal, castrated, adrenalectomized, and hypophysectomized rat. The "escape" as manifested by increasing urinary nitrogen excretion and decreasing weight gain during treatment, as well as the "rebound" in urinary nitrogen excretion with a decrease in weight when the testosterone administration is stopped, occurs in the thyroidectomized rat just as it does in the normal(11), castrate(3), and adrenalectomized(3) rat. However, the action of testosterone in the thyroidectomized rat differs in one respect from that of the normal, castrated, adrenalectomized, or hypophysectomized rats. In the latter groups of animals the decrease of urinary N excretion occurs within 24 hours following the start of treatment, whereas this decrease in the thyroidectomized animals was not evident until 3 days after the start of treatment. The reason for this delayed effect was not evident, but may be due to a decreased rate of absorption of the hormone in the hypothyroid animals.

As was shown previously(6), small, physiologic doses of thyroxin induce protein anabolism (N retention) in the thyroidectomized, but not in the hypophysectomized rat. This fact, together with the observation that thyroidectomy causes loss of eosinophilic granulation in the anterior pituitary and that the eosinophilic granulation is restored by treatment with thyroid hormone(12), suggests that the protein anabolic effect of thyroid hormone may be an indirect effect exerted by inducing growth hormone secretion of the anterior pituitary. The action of testosterone on the other hand must be a direct one, mediated neither through the anterior pituitary nor through the thyroid.

Conclusions. Testosterone propionate after an initial latent period of 3 days causes a decrease in the urinary nitrogen excretion associated with an increased weight gain in the thyroidectomized rat fed a constant intake. Escape during continued treatment, and a "rebound" after the cessation of treatment occurs in the thyroidectomized rat just as it does in the normal, castrated or adrenalectomized rat.

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Physiological Disposition of 2-Phenyl Quinoline-4-Carboxylic Acid (Cinchophen) in Man. Method for its Estimation in Biological Material. (21113)

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Cinchophen (2-phenyl quinoline-4-carboxylic acid) has been extensively used as an analgesic, antipyretic and uricosuric agent. However, its use has diminished in recent years because fatal hepatitis has occasionally occurred during therapy with this drug. In spite of the once widespread use of cinchophen, relatively little information exists concerning its physiological disposition and fate in man. The development of a specific and sensitive method for the estimation of cinchophen in biological material has made it possible to study the fate of the drug in man. Data concerning the absorption, excretion, rate of biotransformation and distribution will be presented in this paper.

Methods. Estimation of cinchophen in biological materials. Cinchophen is isolated from biological materials by extraction into benzene at pH 4.0. The drug is returned to pH 9.0 buffer, acidified and assayed spectrophotometrically at 345 mμ. *Procedure for plasma.* To one to 3 ml plasma in a 60-ml glass-stoppered bottle add an equal volume of 0.4 M citric acid and 30 ml benzene* containing 1.5% isoamyl alcohol.* Shake for 10 minutes and centrifuge the bottle. Transfer 20 ml of the benzene phase to another 60-ml glass-stoppered bottle containing 5 ml of borate buffer pH 9.0 and shake for 5 minutes. Transfer 4 ml of the aqueous phase to a cuvette; add 1.0 ml of 1N HCl and read the optical density at 345 mμ in a Beckman spectrophotometer. Reagents run through the above procedure are used for the zero setting. Standards are prepared by handling a known amount of cinchophen in the same manner as unknown samples. An optical density of 0.103 is obtained in a Beckman spectrophotometer when 10 μg of cinchophen are carried through the procedure. *Procedure for urine.* Proceed as with plasma, but wash the benzene extract 3 times with 1/5th volumes of pH 4.0 buffer.[†] *Procedure for tissues and feces.* Adjust 3 ml of homogenized tissue or feces[‡] to pH 4.0 and proceed as described for plasma. Tissue and red cells contained normally occurring blank material which absorbed light at 345 mμ. Part of this material was removed by washing the benzene extract twice with 1/5th volume of pH 4.0 citrate buffer. The correction for the residual blank depended on the observation that its optical density at 345 and 410 mμ was essentially the same, whereas that of cinchophen was decreased by about 95%. The cinchophen concentration in the solution can be calculated by the following equation: $\frac{\mathrm{Ua}-\mathrm{Ub}}{\mathrm{Sa}-\mathrm{Sb}} \times \mathrm{C}$. Where Ua and Ub are the optical densities of the unknown at 345 and 410 mμ, respectively, Sa and Sb are the optical densities of the standard at these wave lengths and C is the concentration of cinchophen in the standard. Cinchophen added to biological materials in amounts of from 5 to 100 μg was recovered with adequate precision (97 ± 4%). *Assay of specificity.* An examination of specificity for the estimation of cinchophen was made by the technic of comparative distribution ratios (1). Plasma and urine of a human subject who had received the drug were extracted in the same manner as described in the procedure. The partition coefficients of authentic cinchophen between benzene and a number of aqueous solutions at various pH values were compared with those of the apparent compound obtained from biological materials. The results showed that within experimental error the apparent and authentic compounds had the same solubility characteristics and were presumably the same compounds (Table I).

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* Solvents (Reagent grade) are purified by successive washings with 1N NaOH, 1N HCl, and 3 washings with water.

TABLE I. Distribution of Cinchophen and Apparent Cinchophen between Benzene and Water at Various pH Values. The apparent cinchophen from plasma and urine of a subject who had received cinchophen orally was extracted into benzene at pH 4. The urine extract was washed 3 times with pH 4.0 buffer. Aliquots of the benzene extracts were shaken with one-half volume of several buffer solutions. Authentic cinchophen in benzene was treated in the same manner. The fraction of the compound extracted at various pH values is expressed as the ratio of the amount of compound in the organic phase to total compound.

pH	Authentic cinchophen	Apparent cinchophen	
		From plasma	From urine
5.0	.83	.83	.83
5.7	.65	.65	.64
6.2	.45	.47	.45
7.0	.16	.16	.17

Results. Absorption and excretion of cinchophen. Information concerning the absorption from the gastrointestinal tract and renal excretion of cinchophen was obtained from a balance study on 2 human subjects who received 500 mg of the drug orally. Urine and feces were collected over a 72-hour period and examined for cinchophen. Less than 1% of the drug was found in the feces, indicating that absorption from the gastrointestinal tract was essentially complete. The urinary excretion accounted for only 2% of the total dose, indicating that the drug was almost completely metabolized in the body.

Plasma concentration—time curves. Plasma concentrations of cinchophen were measured at various time intervals in 4 subjects who received single oral doses of 500 mg of the drug. Peak plasma levels were achieved in 1.5 to 3 hours after the drug was administered and then declined exponentially (Fig. 1). The biologic half-life of cinchophen, that is, the time required for the plasma drug level to decline by one-half, ranged from 4.0 to 4.5 hours, indicating a relatively uniform rate of biotransformation of the drug in these individuals. The rate of biotransformation of cinchophen is slower than other analgesic drugs such as acetanilide(2), Acetophenetidin (3), and Aminopyrine(4), but faster than antipyrine(5).

[†] The buffer wash serves to remove interfering material which absorbs light at 345 mμ.

[‡] Organ tissues and feces are prepared for analysis by emulsification in acid as described previously(1).

Distribution of cinchophen. The degree to which cinchophen in plasma is bound to proteins was determined by dialysis against isotonic phosphate buffer of pH 7.4 at 37°C for 20 hours. Visking membranes were used for the dialysis bags. A definite relationship between plasma concentration of cinchophen and the degree of binding to the non-diffusible constituents of the plasma was found. At plasma concentrations of 10 mg per liter only 5% of the drug was present in the unbound form, whereas 27% of the compound was found free in the plasma at concentrations of 250 mg/liter. The distribution of cinchophen was examined in representative tissues of a dog which received 150 mg/kg of the drug orally. Two hours after drug administration, the animal was sacrificed by an intravenous injection of air and the tissues immediately sampled (Table II). With the exception of bile, the cinchophen concentration was highest in plasma. The amount of drug present in the plasma water was considerably less because of the high degree of drug binding to plasma protein. Although the concentration of the drug in cerebrospinal fluid was low, the concentration was of the same order of magnitude as that in the plasma water suggesting that there is little hindrance to the passage of drug across the blood-brain barrier. The concentration of cinchophen in most organ tissues was about half that of plasma. However, drug concentrations in organ tissues was higher than that of the plasma water, indicating definite localization of cinchophen in the tissues. The concentration of cinchophen in red cells was about one-fourth that found in plasma, suggesting that the drug is distributed to a considerable degree in the extracellular fluid.

TABLE II. Distribution of Cinchophen in Dog Tissues. The dog received 150 mg/kg of cinchophen orally. The tissues were examined 2 hours after administration of the drug.

Tissue	Conc. of cinchophen (mg/kg)
Plasma	340
Red cells	92
Cerebrospinal fluid	40
Bile	900
Liver	215
Lung	150
Muscle	79
Kidney	168
Heart	134
Brain	42
Spleen	90

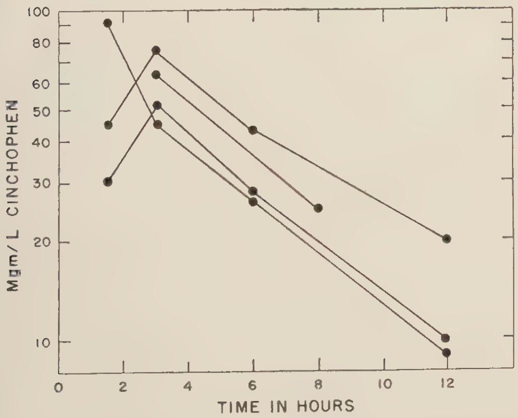

FIG. 1. Plasma levels of cinchophen in man after oral administration of the drug to 4 subjects.

Summary. A specific and sensitive method for the measurement of cinchophen in biological fluids and tissues is described. Cinchophen is essentially completely absorbed from the gastrointestinal tract. The drug is almost entirely metabolized in man, only about 2% being excreted in the urine. The rate of biotransformation of cinchophen is fairly uniform, among a number of subjects the average half-life being 4 hours. Cinchophen is localized in most organ tissues. The drug is largely distributed in extracellular fluid and at low concentrations is highly bound to plasma proteins.

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Persistence of Eosinophil Rhythm after Splenectomy in Rats.* (21114)

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The 24-hour periodicity in number of circulating blood eosinophils seems to be a feature of the physiology of several mammals and the literature on this subject has been discussed(1). It is the purpose of this report to suggest that the eosinophil rhythm may persist in the absence of the spleen.

Materials and methods. Male, Line 3 Black rats, maintained in the Department of Biochemistry at the St. Paul Campus of the University of Minnesota were used. From weaning and throughout the study Purina Fox Chow and tap water were available to the rats *ad libitum*. For 3 weeks prior to as well as during the study the rats were kept singly housed in a room maintained at 25.5 ± 0.5°C; this room was kept illuminated from 06 to 18 and dark from 18 to 06. The rats were 5 months of age at the time of operation and showed no sign of disease. Splenectomy was carried out on 12 rats, ether anesthesia being used. Another group of 12 rats was subjected to a sham-operation. Two independent subgroups, composed of 6 rats each, from the splenectomized group as well as from the sham-operated group were used for the sampling of blood at the 2 times of day chosen for investigation; namely, between 09 and 11 (day) and between 21 and 23 (night). The procedures employed for eosinophil counts have been described(2).

Results. It may be seen from Fig. 1, first, that on the average the day counts were consistently higher than the corresponding night counts, for groups of unoperated or sham-operated rats as well as for splenectomized rats. It is further apparent from Fig. 1 that the mean eosinophil counts of both operated groups show an increasing trend up to 77 days after operation; the mean counts are again lower at 112 days after operation. It may be finally seen from Fig. 1 that the extent of postoperative eosinophilia is larger for rats subjected to splenectomy, in comparison to rats subjected to a sham-operation.

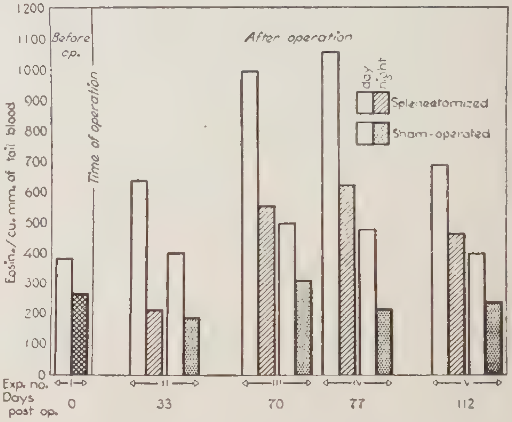

FIG. 1. Eosinophil levels at 2 times of day on Line 3 Black rats, some splenectomized, others subjected to a sham-operation. Note the persistence of eosinophil rhythm after operations.

Discussion. The observation of eosinophilia following spleen removal is in keeping with corresponding earlier observations on several species, including sporadic observations on man(3), except for the earlier development and earlier regression of the phenomenon in the rat. Species differences in the times of development and of regression of eosinophilia after splenectomy may be related to differences in life span. The times of development and of regression of changes in eosinophil level, as noted herein, agree well with earlier data on changes in the levels of other circulating leukocytes, after splenectomy on rats(4).

The observation that the day-night differences in mean eosinophil count of rats persist for several months after splenectomy is in keeping with the suggestion that the presence of the spleen is not essential for the maintenance of the eosinophil rhythm, at least as far as the rats studied are concerned.

Summary. Eosinophil counts were made on tail blood obtained at two times of day from

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male Line 3 Black rats kept under standardized circumstances and subjected either to splenectomy or to a sham-operation. Eosinophilia was seen to develop during the first few months after operation and to regress during the fourth month after operation. Irrespective of the degree of eosinophilia, the mean eosinophil counts of the groups of rats studied were higher by day than by night. In the strain of rats studied, the presence of the spleen is not essential for the maintenance of the eosinophil rhythm.

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Serologic Characterization of the Fort Bragg Leptospire. (21115)

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Outbreaks of a disease that appeared among soldiers stationed on the military reservation of Fort Bragg, N. C. during the summer months of 1942, 1943, and 1944, became known as Fort Bragg or Pretibial Fever(1,2). The infectious agent, isolated by Tatlock in 1944 from the blood of an acutely ill patient and subsequently maintained in various laboratories by serial animal passages, was believed to be a virus until Gochenour *et al.*(3) clearly established the leptospiral etiology of Fort Bragg Fever, and recovered the leptospiral agent from infectious material of the 259th serial hamster passage. Preliminary serologic studies by these workers by means of agglutination-lysis procedures indicated that the Fort Bragg agent was related to the type strain *Leptospira autumnalis,* Akiyami A. This was the first report of a member of the *L. autumnalis* serogroup in the United States. *Classification of leptospires* is based upon stable and specific antigenic characteristics revealed by the agglutination-lysis reaction(4). The genus is divided into "serogroups" of closely related "serotypes." By definition, a serotype includes all strains indistinguishable one from the other on the basis of cross-absorption procedures. Within the serogroup, however, one may find in addition to heterologous serotypes, "complete" and "incomplete" biotypes. These latter may be designated by the antigenic symbols AB and A respectively, indicating that the "incomplete" biotype contains part, but not all of the antigenic components of the "complete" biotype(5). Wolff's(5) proposed classification of leptospires, based upon their cross-agglutination-lysis reactions, groups 36 antigenically distinct leptospiral serotypes into 20 serogroups. This scheme has been tentatively accepted by various leptospiral typing laboratories throughout the world as a working basis in the typing of leptospiral strains. The *autumnalis* serogroup listed in Wolff's scheme is comprised of the following serotypes:

1. *Autumnalis AB* (Type strain Akiyami A) Etiologic agent of autumnal fever in Japan and frequently found in S. E. Asia. 2. *Autumnalis A* (type strain Rachmat). Incomplete "biotype" of *autumnalis AB,* found in S. E. Asia. 3. *Bangkinang* (type strain Bangkinang I). A heterologous member of the *autumnalis* group found in S. E. Asia. With the recent acquisition of the Rachmat and Bankinang strains, an antigenic analysis of the Fort Bragg leptospire was performed using the agglutination-lysis technic.

Methods. Preparation of immune serums. Normal rabbits weighing approximately 3 kg were inoculated intravenously with 1.0 ml, 2.0

TABLE I.
Cross Agglutination-Lysis Reaction of the Fort Bragg and *Autumnalis* Serogroup Strains.

Immune sera	Homologous titer	Antigens			
		Fort Bragg	Akiyami A	Rachmat	Bangkinang I
	×100				
Fort Bragg	1024	1024	1024	256	1024
Akiyami A	1024	1024	1024	64	256
Rachmat	4096	1024	4096	4096	64
Bangkinang I	256	256	256	64	256
andaman	256	—	—	—	—
semarang	256	—	—	—	—
djatzi*	256	—	—	—	—
hyos	256	—	—	—	—
pomona	64	1	1	1	1
djasiman	256	1	—	1	—
sentot	256	16	64	4	16
ballico	64	—	—	1	—
ballum	256	—	—	—	—
canicola	1024	—	—	—	—
ictero. AB	256	1	—	1	—
pyrogenes	256	—	—	—	—
alexi†	1024	—	—	—	—
grippotyphosa	4096	16	—	4	4
borincano‡	256	—	—	—	—
sejroe	1024	—	—	—	—
javanica	256	—	—	—	—

* Member of the serogroup *bataviae.*
† " " " " *pyrogenes.*
‡ " " " " *hebdomadis.*

ml, 4.0 ml, and 6.0 ml of a 5- to 7-day-old live Fletcher's(6) culture of the desired leptospiral strains at 5- to 7-day intervals. Five to 7 days following the last inoculation, the rabbits were exsanguinated and the serums harvested. The serums after Seitz filtration were stored at –20°C. *Antigens employed.* Five- to 7-day-old Schuffner's(7) cultures containing live leptospires were employed as antigens. *Agglutination-lysis test.* Serial 4-fold dilutions were prepared with each serum to be tested using buffered saline (pH 7.4) as diluent and providing serum dilutions of from 1:50 through 1:204,800. To 0.2 ml of each serum dilution in a standard Kahn tube was added an equal quantity of antigen. The tubes were shaken, incubated at 30°C for 3 hours and then examined by (10 X objective and 15 X ocular) dark-ground illumination without the use of cover glasses. Agglutination or lysis, or both, were read as complete, partial, trace, or absent. Titers were expressed as the reciprocals of the highest dilution in which complete agglutination or lysis was observed. *Absorption procedure.* Absorbing antigen was prepared from 7- to 9-day-old leptospiral cultures maintained in Stuart's medium(8) (without glycerol) at 30°C. Cultures were centrifuged at approximately 4000 g in the International Centrifuge PR-1 for one hour in 100 ml cups employing International Centrifuge head No. 845. The supernatant fluid was discarded and the leptospires were resuspended to approximately 1% of the original culture volume with 0.25% formalinized buffered saline (pH 7.4). Four parts of the absorbing antigen were added to one part of a 1:10 dilution of immune serum, placed in a shaker apparatus for two hours, and maintained overnight in a 30°C incubator. In order to avoid further dilution of the serum, subsequent absorptions were performed with centrifuged packed leptospires, obtained by centrifuging the same quantity of antigen suspension used in the first absorption, at approximately 20,000 g for 10 minutes in the International centrifuge PR-1 using 7.0 ml cups and the high speed adapter head, and discarding the supernatant. The absorbing mixtures were centrifuged in the same manner, the supernatant withdrawn and tested by the described agglutination-lysis procedure. Routinely two successive absorptions were performed. Absorptions were considered com-

TABLE II. Cross Agglutinin-Absorptions.

Antiserum		Antigen			
Against	Absorbed with	*aut.* Akiyami A	*aut.* Rachmat	Bangkinang	Fort Bragg
		× 100			
1. *autumnalis* AB Akiyami A	Unabsorbed	256	16	64	256
	1 *aut.* Aki. A†	—	—	—	—
	2 *aut.* Rachmat	64	—	16	64
	3 Bangkinang	256	1	—	64
	4 Fort Bragg†	4	—	—	—
2. *autumnalis* A Rachmat	Unabsorbed	64	64	16	64
	1 *aut.* Aki. A*	—	4	—	—
	2 *aut.* Rachmat	—	—	—	—
	3 Bangkinang	—	4	—	—
	4 Fort Bragg*	—	4	—	—
3. Bangkinang	Unabsorbed	64	16	64	64
	1 *aut.* Aki. A	1	1	16	4
	2 *aut.* Rachmat	64	—	64	64
	3 Bangkinang	—	—	—	—
	4 Fort Bragg*	1	1	16	—‡
4. Fort Bragg	Unabsorbed	64	16	16	256
	1 *aut.* Aki. A†	—	1	1	16
	2 *aut.* Rachmat	16	—	16	256
	3 Bangkinang*	16	1	—	64
	4 Fort Bragg†	—	—	—	—

* Absorbed 3 times.
† " 4 " .
‡ 1st dilution not done, 2nd dilution neg.

plete when no macroscopic agglutination was evident in the absorbing mixture and agglutinins were completely removed by the homologous antigen. If necessary, additional absorptions were performed with packed cells.

Results of the cross agglutination-lysis reactions of the Fort Bragg isolate and members of the serogroup *autumnalis* employed as antigens against a screening battery of type-leptospiral immune rabbit serums, selected on the basis of previous serologic classification studies (9) is shown in Table I. Within the limitations of this procedure, the Fort Bragg strain is indistinguishable from other strains of the *autumnalis* serogroup. However, subsequent antigenic analysis utilizing agglutinin-absorption procedures (Table II) clearly demonstrates that these strains are distinctly heterologous, sharing common major antigens. The agglutinins responsible for the strain specificity are not completely removed when absorbed with any of the other strains, although in several instances there are marked reductions in homologous titers. It is interesting to note that absorption of the Rachmat strain (the "incomplete" biotype) agglutinins with Akiyami A (the "complete" biotype) antigen does not affect a complete reduction of homologous titer.

Discussion. According to Wolff's proposed scheme of leptospiral classification(5) a serogroup is comprised of sterotypes sharing major antigenic components. Wolff "provisionally considered two strains to be heterologous if the antiserum of each strain, after absorption by the other strain, retains at least 10% of its original titer when retested against the homologous strain." This same criterion must necessarily apply to the consideration of "incomplete" and "complete" biotypes.

The leptospiral strains like the members of the genus *Salmonella* show marked serologic heterogeneity. The application of an arbitrary numerical standard may have particular merit in the determination of serotypes especially in view of the fact that the relatively low yield of leptospiral cultures practically precludes any comprehensive antigenic factor analysis. There are, however, certain drawbacks in Wolff's scheme to simplify leptospiral classification, attributable to the many variable factors inherent in agglutination-lysis absorption procedures which affect titers. Quantitative differences in titers may be markedly affected by the use of formalinized or living cultures, by the age and density of antigens employed in agglutination-lysis procedures(10), by the maximum degree of agglutination and/or lysis accepted as positive and by the particular immune rabbit serum employed(11). It is to be expected that different laboratories employing diversified technics may differ quantitatively in the antigenic evaluation of strains. Until a suitable stable antigen is developed and incorporated into a universally acceptable standardized agglutination-lysis procedure, we have applied Wolff's proposals, with some modifications, to the study of leptospiral serotypes.

The agglutinin-absorption technics employed by Wolff and other European workers differ from those used at the Army Medical Service Graduate School in their initial dilution of all immune serums to a standard titer of 3000, in their employment of a final dilution scheme of 1:10, 1:30, 1:100, 1:300, 1:1000, and 1:3000 and in their use of formalinized test antigens(12). Thus with their dilution scheme, 2 strains are considered homologous if the antiserum of each strain, after absorption by the other strain, is reduced to at least 3⅓% of its original homologous titer, while the retention of at least 10% of the homologous titer is the criterion for heterologous strains. With the technic employed in our studies, we have adopted 1.6% (1/64 of homologous titer) and 6.2% (1/16 of homologous titer) as the breakpoints in the determination of homologous and heterologous serotypes. Our selection of these criteria particularly in view of the serial dilutions employed, is based on numerous absorption studies, and in most of these studies the results have been compatible with the findings of the European workers.

The application of these criteria to the cross agglutinin-absorption results in Table II indicates that the *autumnalis*, Akiyami A strain is an "incomplete" biotype of the Fort Bragg isolate and that contrary to previous reports (5) the Rachmat strain is a heterologous member of the *autumnalis serogroup*. We propose the designation of the Fort Bragg isolate as *L. autumnalis*, Fort Bragg.

Summary. 1. Results of cross agglutination-lysis and cross agglutinin-absorption studies indicate that the etiologic agent of Fort Bragg Fever is closely related to the other members of the leptospiral serogroup *autumnalis*. 2. Agglutinin-absorption studies with all members of the serogroup *autumnalis* reveal that the agglutinins responsible for strain specificity are not completely removed when absorbed with any of the other strains. 3. Classification of these leptospiral strains on the basis of a modified adoption of Wolff's scheme(5) designates *autumnalis* Akiyami A as the "incomplete" biotype of the Fort Bragg isolate and discloses *L. autumnalis A* Rachmat, in addition to *L. bangkinang*, as a heterologous serotype member of the autumnalis group. 4. The Fort Bragg isolate is therefore designated as *L. autumnalis*, Fort Bragg.

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